Dose-dependent Levels of Epigallocatechin-3-gallate in Human Colon Cancer Cells and Mouse Plasma and Tissues

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Running title: Dose-dependent levels of EGCG *in vitro* and *in vivo*

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Abbreviations: area under the curve, AUC; maximum concentration, C_{max}; catechol-*O*-methyltransferase, COMT; epicatechin-3-gallate, ECG; epigallocatechin-3-gallate, EGCG; monocarboxylate transporter, MCT; multidrug resistance related protein, Mrp; UDP-glucuronosyltransferase, UGT

Abstract

Epigallocatechin-3-gallate (EGCG, mol. formula: $C_{22}H_{18}0_{11}$) is the most abundant catechin in green tea (*Camellia sinensis* Theaceae). Both EGCG and green tea have been shown to have cancer preventive activity in a number of animal models, and numerous mechanisms have been proposed based on studies with human cell lines. EGCG has been shown to undergo extensive biotransformation to yield methylated and glucuronidated metabolites in mice, rats, and humans. In the present study, we determined the concentration-dependent uptake of EGCG by HT-29 human colon cancer cells (20 – 600 µM) and the dose-dependence of EGCG plasma and tissue levels following a single dose of EGCG (50 – 2000 mg/kg, *i.g.*) to male CF-1 mice. The cytosolic levels of EGCG were linear with respect to extracellular concentration of EGCG following treatment of HT-29 cells for 2 h (915.3 – 6851.6 µg/g). *In vivo* EGCG exhibited a linear dose-relationship in the plasma (0.03 – 4.17 µg/mL), prostate (0.01 – 0.91 µg/g), and liver (0.09 – 18.3 µg/g). In the small intestine and colon, however, the levels of EGCG plateaued between 500 and 2000 mg/kg, *i.g.* These results suggest that absorption of EGCG from the small intestine is largely via passive diffusion, however, at high concentrations, the small intestinal and colonic tissues become saturated. The levels of 4"-*O*-methylEGCG and 4',4"-di-*O*-methylEGCG parallel those of EGCG with respect to dose. The present study provides information with respect to what concentrations of EGCG are achieveable in mice and may guide dose-selection for future cancer chemoprevention studies with EGCG.

Introduction

Epigallocatechin-3-gallate (EGCG, Fig. 1) is the most abundant and widely studied

catechin in green tea (*Camellia sinensis* Theaceae). Both green tea and EGCG have been

reported to have cancer preventive activities in a number of animal models (Yang et al.,

2002, Lambert and Yang, 2003). Numerous potential mechanisms have been proposed to

account for these activities including: inhibition of growth factor receptor signaling,

inhibition of activator protein-1 and nuclear factor kappa B-mediated transcription,

inhibition of the proteasome, and others (Yang et al., 2001, Nam et al., 2001, Liang et al.,

1997, Masuda et al., 2001). In most studies, the concentrations needed to observe these

activities typically range from $1 – 100$ μM: concentrations which exceed those found in

rodent and human plasma by $10 – 100$-fold (Lambert and Yang, 2003).

Chow *et al.* have previously reported, in one of the only careful dose-finding

studies, that following oral administration of EGCG to humans, a linear increase in

plasma concentrations and area under the curve (AUC) was observed over a dose range

of $200 – 600$ mg (Chow et al., 2001). Administration of 800 mg EGCG, however,

resulted in a greater than predicted plasma C_{max} and AUC. The authors hypothesized that

this increase might be due to saturation of factors (*e.g.* phase II enzymes, efflux pumps,

etc.) which limit bioavailability of EGCG.

EGCG has been show to undergo extensive phase II metabolism including

glucuronidation and methylation *in vitro* and *in vivo* (Lu et al., 2003b, Lu et al., 2003a,

Lambert et al., 2003). We have previously reported on the active efflux of EGCG by

multidrug resistance related protein (Mrp)1 and 2 from human cancer cell lines *in vitro*

(Hong et al., 2003). Additionally, epicatechin-3-gallate (ECG) has been reported to be a

substrate of monocarboxylate transporters (MCT) (Vaidyanathan and Walle, 2003, Hong et al., 2003). These represent potential saturable factors, which may limit absorption of EGCG from the gut and its availability to the plasma, and may explain the phenomena observed by Chow *et al* (Chow et al., 2001).

We have previously determined the pharmacokinetic parameters of EGCG in the mouse and ascertained that this species is more similar to humans in terms of EGCG bioavailability than is the rat (Lambert et al., 2003). In the present study, we examined the dose-dependency of plasma and tissues levels of EGCG in mice. Based on energy consumption calculations, the doses selected (50 – 2000 mg/kg, *i.g.*) include the range used by Chow *et al.* in their study and extend the range by 10-fold greater dose. Herein, we report the results of our study.

Materials and Methods

Chemicals

EGCG (100% pure) was provided by Tokyo Food Techno Co. (Fujieda City, Japan). β-D-glucuronidase (G-7896, EC 3.2.1.31, from *Escherichia coli* with 9 x 10^6 U/g solid) and sulfatase (S-9754, EC 3.1.6.1, from Abalone entrails with 2.3 x 10^5 U/g solid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade commercially available. Dosing solutions of EGCG were prepared in 0.9% NaCl containing 15% ethanol. For analytical purposes, a standard stock solution of EGCG, epigallocatechin, epicatechin, and epicatechin-3-gallate (ECG) (10 µg/mL each) was prepared in 0.2% ascorbic acid-0.005% EDTA (pH 3.8) and stored at –80°C.

Cell Culture and Treatment

HT-29 human colon adenocarcinoma cells (American Type Culture Collection, Rockville, MD) were maintained in log-phase growth in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 95% relative humidity and 5% CO_2. Dose-dependent cellular uptake of EGCG over a dose range of 20 – 600 µM for 2h was determined using previously described methods (Hong et al., 2003). In brief, once cells reached 70% confluence in 6-well plates, medium was replaced with Hank's Balanced Salt Solution (HBSS, pH 7.4). Cells were allowed to equilibrate for 2h, then fresh HBSS containing 100 mM ascorbic acid and the desired concentration of EGCG was added. Following incubation at 37°C for 2h, cells were washed 3 times with PBS and scraped into 2% ascorbic acid. The cells were sonicated with a probe sonicator and centrifuged at 14,000

rpm to pellet cell debris. The supernatant (*i.e.* cytosol) was transferred to a new tube.

The protein content was determined by the method of Bradford, and the superntant was

combined with an equal volume of ice-cold methanol. Following centrifugation, 50 μL

of the supernatant was analyzed by HPLC with electrochemical detection.

Animals

Male CF-1 mice (30 – 35 g) were purchased from Charles River Laboratories

(Wilmington, MA, USA) and allowed to acclimate from at least one week prior to the

start of the experiment. The mice were housed 10 per cage, and maintained in air-

conditioned quarters with a room temperature of $20 \pm 2°C$, relative humidity of $50 \pm 10\%$,

and an alternating 12 h light/dark cycle. Animals were given Purina rodent chow

(Research Diets, New Brunswick, NJ, USA) and water *ad libitum* and fasted overnight

prior to treatment.

Treatment and Sample Collection

Following overnight fast, mice (six per group) were given a single dose of EGCG (50 –

2000 mg/kg, *i.g.*) and sacrificed at 50 or 180 min. Blood was collected from anesthetized

animals by cardiac puncture and plasma was isolated by centrifugation at 500 x *g* for 15

min. Plasma was combined with a $1/10^{th}$ volume of ascorbate preservative (20% ascorbic

acid-0.1% EDTA) and stored at $-80°C$ for later analysis. The lungs, liver, colon, small

intestine, and prostate were collected, washed in 0.9% NaCl, and frozen at $-80°C$ for later

analysis.

Quantification of EGCG and Metabolites

Plasma levels of EGCG and its metabolites were analyzed as previously reported (Chen et al., 1997). In brief, 100 μL of plasma was hydrolyzed with 1 U sulfatase, and 250 U β-glucuronidase at 37^oC for 45 min, and extracted methylene chloride and ethyl acetate. The ethyl acetate fractions were pooled and dried under vacuum. Samples were reconstituted in 10% aqueous acetonitrile and analyzed by HPLC.

Tissue samples were prepared and analyzed by our standard methods (Lambert et al., 2003). In brief, samples were homogenized in 2 volumes of ice-cold 2% ascorbic acid using a mechanical dounce homogenizer and 200 μL aliquots were hydrolyzed, extracted, and analyzed in a manner identical to plasma. EGCG and its methylated metabolites were identified by comparing samples with the retention times of authentic standards. Quantification was based on comparison of peak heights with standard plasma containing a known amount of compound.

Sample Analysis

EGCG levels were analyzed using an HPLC consisting of two ESA model 580 dual-piston pumps (Chelmsford, MA, USA), a Waters Model 717plus refrigerated autosampler (Milford, MA, USA), and an ESA 5500 coulochem electrode array system (CEAS). The potentials of the CEAS were set at –100, 100, 300, and 500 mV. Separation was achieved using a binary mobile phase of solvent A ($0.03M$ NaH_2PO_4, pH 3.35, containing 1.75% acetonitrile and 0.125% tetrahydrofuran) and solvent B (0.015 M NaH_2PO_4, pH 3.45, containing 58.5% acetonitrile and 6.25% tetrahydrofuran as previously reported (Chen et al., 1997).

Results and Discussion

Incubation of HT-29 cells, a commonly used cell line for drug uptake studies, with concentrations of EGCG ranging from 20 – 600 μM resulted in a linear increase in the cytosolic concentration of EGCG (Fig. 1). Although we have previously shown that these cells express Mrp pumps capable of effluxing EGCG from the cytosol and UDP-glucuronosyltransferase (UGT) and catechol-*O*-methyltransferase (COMT) activity capable of biotransforming EGCG (Hong et al., 2002), our present results suggest that these are not saturated under the present conditions.

To extend these *in vitro* studies, we examined the dose-concentration relation of EGCG following intragastric administration of EGCG to CF-1 mice. The doses used are approximately equivalent to consumption of 2 – 53 cups of green tea (185 mg EGCG per cup) by a 70 kg person. The dose conversion from humans to mice was based on scaling dose to daily caloric intake (*i.e.* 2000 kcal/d for humans and 12 kcal/d for mice) using the following equation (Schneider et al., 2004):

From mg/kg in human to mg/kcal:

$$(Dose_{mg/kg} * body\ weight_{kg})/2000\ kcal = Dose_{mg/kcal}$$

From mg/kcal to mg/kg in mice:

$$(Dose_{mg/kcal} * 12\ kcal)/body\ weight_{kcal} = Dose_{mg/kg}$$

No acute toxicity was observed following single dose administration of up to 2000 mg/kg, *i.g.* A linear increase in the plasma concentration of EGCG was observed 50 and 180 min after administration of 50 – 2000 mg/kg, *i.g.* EGCG to male CF-1 mice (Fig. 2). The concentration of total and unconjugated EGCG in the plasma ranged from 0.03 – 4.17 μg/mL and 0.01 – 0.37 μg/mL, respectively. Plasma levels were higher at 50

min than at 180 min and EGCG was largely conjugated as the glucuronide (45 – 90%):

these observations are consistent with our previous pharmacokinetics study in the mouse

(Lambert et al., 2003). There was no apparent relationship between dose and the

percentage of conjugated versus unconjugated EGCG. Interestingly, we did not observe

the greater than linear increase in the plasma concentrations of EGCG as was previously

reported in humans at high doses (Chow et al., 2001). It is unclear whether this is a

species difference or is the result of the statistical variability in human studies.

The concentration of EGCG in the prostate and liver also showed a linear

relationship to dose (Fig. 2). EGCG at doses of 50 – 2000 mg/kg, *i.g.* resulted in liver

and prostate concentrations of 0.09 – 18.3 µg/g and 0.01 – 0.91 µg/g, respectively.

Prostate concentrations for most doses were below the limit of detection at 180 min (data

not shown). As we have previously reported, EGCG was in the unconjugated form in the

tissues (Lambert et al., 2003). By contrast, levels of EGCG in the small intestine and

colon were curvilinear with respect to EGCG dose (Fig. 2). These levels plateaued

between 500 and 2000 mg/kg, *i.g.* and resulted in maximal concentrations of 16.1 – 162.9

µg/g and 0.5 – 23.4 µg/g in the small intestine and colon, respectively. As we have

previously observed, the levels in the small intestine were higher at 50 min while those in

the colon were higher at the 180 min. The colonic concentration at 180 min after 1000

mg/kg dose was greater than expected. We believe that this point is anomalous and is the

result of incomplete washing of the colon samples in this treatment group. This resulted

in residual fecal matter in the sample, which led to higher than expected levels. The

dose-dependent levels of 4"-*O*-methyl-EGCG and 4',4"-di-*O*-methyl-EGCG mirrored

those of EGCG in the liver (0.05 – 0.49 µg/g and 0.09 – 0.14 µg/g, respectively), the

prostate (0.01 – 0.12 µg/g and 0.002 – 0.03 µg/g), the small intestine (0.32 – 1.74 µg/g

for 4"-*O*-methylEGCG), and the colon (0.04 – 0.27 µg/g for 4"-*O*-methylEGCG).

It is possible that at the higher doses (*i.e.* 500 and 2000 mg/kg, *i.g.*), EGCG has

saturated the small intestinal and colonic tissues, resulting in a plateau of the levels in

these tissues. EGCG continues to cross the intestinal barrier, however, due to the lower

concentrations in the plasma and other tissues and higher saturation point of EGCG in

these tissues: this would manifest as a linear increase in plasma or tissue concentration

with respect to dose. In addition, the linear increase in plasma and other tissues may

represent a saturation of the active elimination of EGCG by the small intestine (via Mrp2)

or the liver (via biliary secretion). Overwhelming hepatic clearance would allow a

greater amount of EGCG to exit the portal circulation and enter the systemic circulation.

Further studies are needed to determine the dose-dependent absorptive capacity of the

small intestine and its saturation point for EGCG. The fact that *in vitro* uptake studies

using HT-29 cells did not predict the saturation of small intestinal and colon levels of

EGCG suggests the limitation of this cell line as a model for the intestinal barrier. Likely

this is because the model fails to take into account the impact of hepatic clearance, the

presence of intestinal mucosal layer, and other factors.

To conclude, we have determined the dose-dependence of EGCG levels in HT-29

human colon adenocarcinoma cells and in CF-1 mice following single-dose oral-

administration of EGCG. Cytosolic EGCG levels were linear with respect to

extracellular concentration *in vitro*. *In vivo*, the plasma, liver, and prostate levels of

EGCG exhibited a linear dose-concentration relationship: the dose-concentration

relationship in the small intestine and colon was curvilinear and plateaued between 500

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and 2000 mg/kg, *i.g.* These doses were not acutely toxic and these data should be useful

in designing future cancer chemoprevention studies.

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factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal

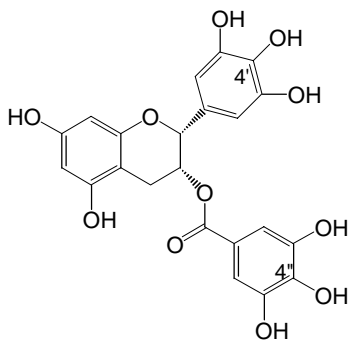epithelial cell line IEC-6. *Mol Pharmacol,* 60**,** 528-33.

Footnotes

Figure Legends

Figure 1.   Dose-dependent cytosolic concentration of EGCG in HT-29 human colon

adenocarcinoma cells. Cells were exposed to 20 – 600 μM EGCG in

HBSS, pH 7.4 for 2h. The cytosolic fraction was prepared and EGCG

levels were determined by HPLC. Each point represents the mean of n =

4. Error bars represent the standard deviation.

Figure 2.   Concentration of total EGCG in the plasma, liver, prostate, small intestine,

and colon of male CF-1 mice 50 or 180 min after treatment with a single

dose of EGCG (50 – 2000 mg/kg, *i.g.*). For doses: 50, 200, 500 and 2000

mg/kg, the points represent the average of two independent experiments

with and overall n = 12. For doses: 100 and 1000 mg/kg, the points

represent the average of one independent experiment with n = 6. Error

bars represent the standard error of the mean.

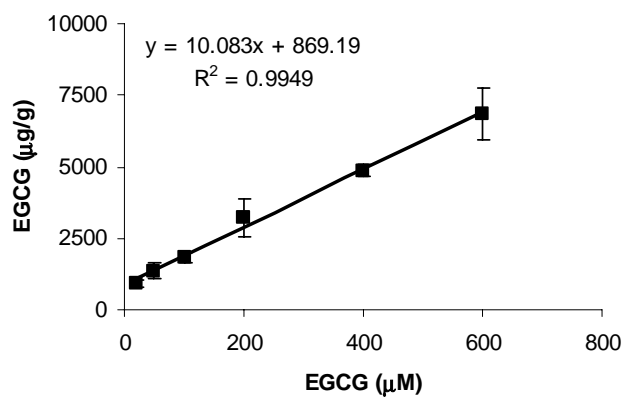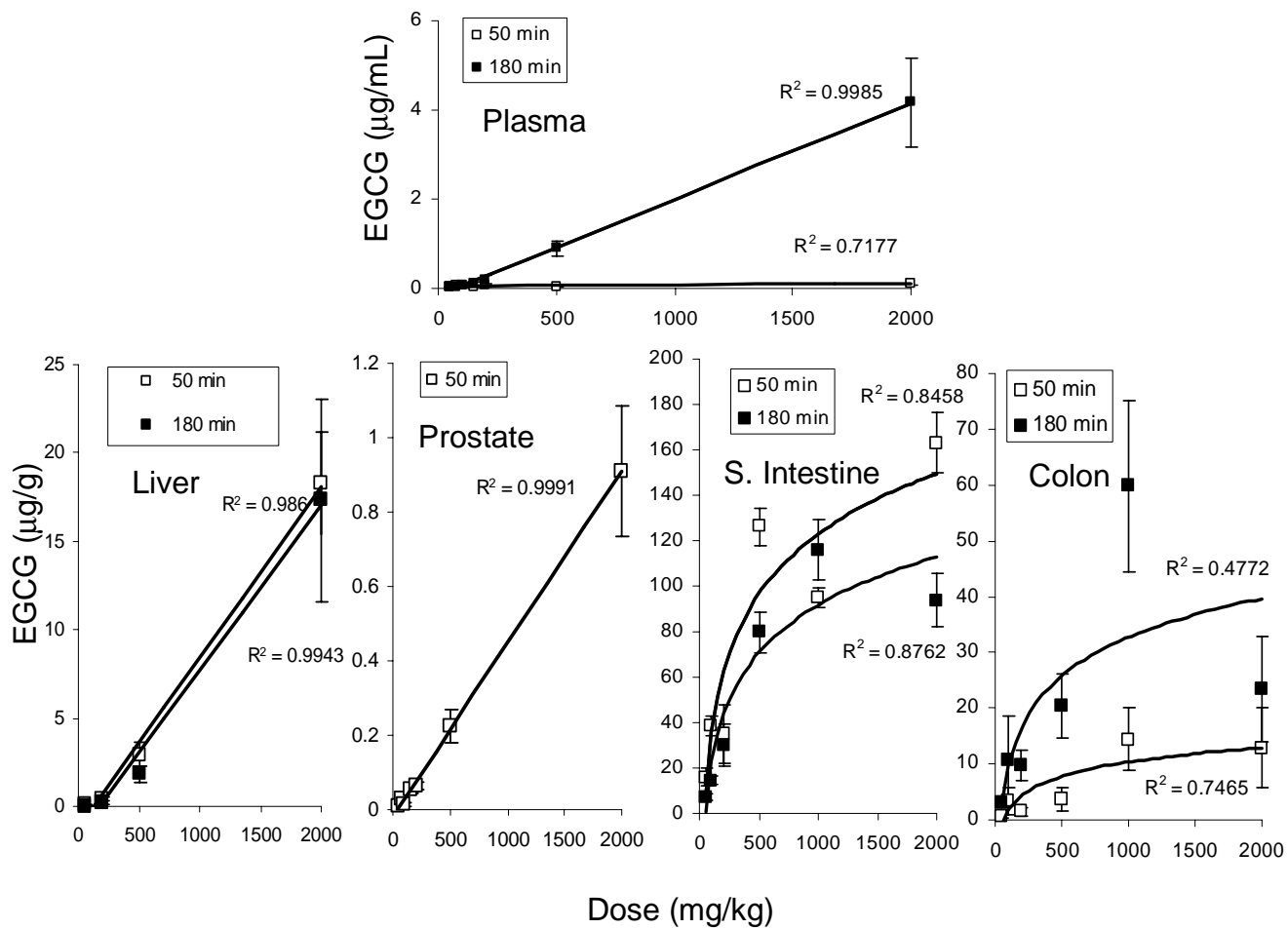Epigallocatechin-3-gallate (EGCG)

$y = 10.083x + 869.19$
$R^2 = 0.9949$

Figure 1

Figure 2