Peracetylation as a Means of Enhancing in Vitro Bioactivity and Bioavailability of Epigallocatechin-3-Gallate

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ABSTRACT:

(−)-Epigallocatechin-3-gallate (EGCG) is the widely studied catechin in green tea (Camellia sinensis). Previously, we have reported the low bioavailability of EGCG in rats and mice. As a means of improving the bioavailability of EGCG, we have prepared a peracetylated EGCG derivative (AcEGCG) and herein report its growth inhibitory activity and cellular uptake in vitro, as well as bioavailability in mice. AcEGCG exhibited enhanced growth inhibitory activity relative to EGCG in both KYSE150 human esophageal (IC50 = 10 versus 20 μM) and HCT116 human colon cancer cells (IC50 = 32 versus 45 μM). AcEGCG was rapidly converted to EGCG by HCT116 cells, and treatment of cells with AcEGCG resulted in a 2.8- to 30-fold greater intracellular concentration of EGCG as compared with treatment with EGCG. AcEGCG was also more potent than EGCG at inhibiting nitric oxide production (4.4-fold) and arachidonic acid release (2.0-fold) from lipopolysaccharide-stimulated RAW264.7 murine macrophages. Intragastric administration of AcEGCG to CF-1 mice resulted in higher bioavailability compared with administration of equimolar doses of EGCG. The plasma area under the curve from 0 to infinity (AUC0 t) of total EGCG was 465.0 and 194.6 ([μg/ml] · min) from the administration of AcEGCG and EGCG, respectively. The t1/2 of EGCG was also increased following administration of AcEGCG compared with EGCG (441.0 versus 200.3 min). The AUC0 t and t1/2 were also increased in small intestinal (2.8- and 4.3-fold, respectively) and colonic tissues (2.4- and 6.0-fold, respectively). These data suggest that acetylation represents a means of increasing the biological potency in vitro, increasing the bioavailability of EGCG in vivo, and may improve cancer-preventive activity.

Green tea (Camellia sinensis, Theaceae) and its catechin components, especially epigallocatechin-3-gallate (EGCG, molecular formula: C22H18O11) (Fig. 1), have shown cancer-preventive activity in a number of animal models (Lambert and Yang, 2003b; Lambert et al., 2005). Numerous mechanisms have been proposed for this activity based on studies of human cancer cell lines, including inhibition of growth factor signaling, inhibition of kinases, inhibition of DNA methyltransferase, and others (Higdon and Frei, 2003; Hou et al., 2004). Because of the low bioavailability of EGCG, it is unclear which of these mechanisms play primary roles in cancer prevention.

We have previously shown that the bioavailability of EGCG in mice and rats is 26.5 and 1.6%, respectively (Chen et al., 1997; Lambert et al., 2003). EGCG has a large number of hydrogen bond donors (i.e., hydroxyl groups), has a large polar molecular surface area, and is predicted by “Lipinski’s Rule of 5” to be poorly absorbed (Clark, 1999; Lipinski et al., 2001). EGCG is subjected to extensive glucuronidation, methylation, and sulfation, as well as to microbial degradation in the colon (Kida et al., 2000; Lu et al., 2003a,b). The small intestine appears to be a major barrier to the bioavailability of EGCG. Cai et al. (2002) found that although the oral bioavailability of EGCG is low in the rat, only a small percentage of EGCG, administered by the portal vein, is metabolized by the liver. This suggests that poor absorption from the small intestine may play an important role in limiting EGCG bioavailability. Similarly, our laboratory has reported that the intestine-specific human UDP-glucuronosyltransferase 1A8 and mouse small intestine microsomes were more active at glucuronidation of EGCG than the liver (Lu et al., 2003a). Cell line studies have also suggested that EGCG and other catechins are subject to active efflux by multidrug resistance-related proteins (Hou et al., 2003; Vaidyanathan and Walle, 2003). Chemical modifications that can improve the physiochemical properties of EGCG or reduce its biotransformation may be useful in improving its bioavailability.

Ester-based prodrugs are classical means to improve the bioavailability and reduce the toxicity of a compound (Liederer and Borchart, 2006). For example, acetylation of salicylic acid to form aspirin accomplishes this, as does acetylation of morphine to produce heroin (Sollman, 1957; Klaassen, 1996). This improvement is accomplished by occlusion of polar side chains, increasing hydrophobicity, and making hydroxyl groups unavailable for phase II biotransformation or oxidative degradation. Previously Lam et al. (2004) have reported that peracetylated EGCG (AcEGCG, molecular formula...
C_{18}H_{13}O_{19} (Fig. 1) represents a prodrug for inhibition of proteasome activity. The authors found that this compound had increased activity against proteasome activity in intact cells but not against pure enzymes.

In the present study, we have examined the in vitro growth inhibitory and anti-inflammatory activity of AcEGCG, the kinetics of AcEGCG de-esterification, and the oral bioavailability of this compound in mice.

### Materials and Methods

**Chemicals.** EGCG (100% pure) was isolated from a crude green tea polyphenol extract and provided by Dr. Chi-Tang Ho (Department of Food Science, Rutgers University). AcEGCG was synthesized using a pyridine-polyphenol extract and provided by Dr. Chi-Tang Ho (Department of Food Science, Rutgers University). AcEGCG was synthesized using a pyridine-polyphenol extract and provided by Dr. Chi-Tang Ho (Department of Food Science, Rutgers University).

**Cell Culture.** KYSE150 human esophageal cancer cells and HCT116 human colon adenocarcinoma cells were plated in 96-well plates (2–5 × 10^3 cells/well) and allowed to attach for 24 h. The medium was replaced with fresh, serum-free medium containing 0 to 40 μM EGCG or AcEGCG. Cells were incubated for 24 h at 37°C. The medium was then replaced with fresh serum-complete medium, and the cells were incubated for an additional 24 h at 37°C. Growth inhibition was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (Bold et al., 2001).

Cellular uptake and cytosolic levels of EGCG and AcEGCG following treatment of HCT116 cells with either AcEGCG or EGCG were determined as previously reported (Hong et al., 2003). In brief, cells were allowed to grow to 70 to 90% confluence in 12-well plates. The medium was replaced with fresh medium containing 20 μM EGCG or AcEGCG and 5 U/ml superoxide dismutase (to stabilize the EGCG). The cells were incubated for 0.25 to 24 h at 37°C, after which the medium was removed, the cells were washed twice with cold phosphate-buffered saline, 2% ascorbic acid was added to each well, and the cells were scraped and sonicated. The resulting solution was centrifuged at 10,000 g for 20 min. The supernatant was combined with an equal volume of ice-cold methanol and centrifuged for 20 min at 10,000g to precipitate the protein. The resulting supernatant was analyzed by high-performance liquid chromatography (HPLC). Cytosolic EGCG was normalized to cytosolic protein concentration.

**Inhibition of Arachidonic Acid Release and NO Production.** The ability of EGCG and AcEGCG to inhibit the release of arachidonic acid and production of NO by lipopolysaccharide (LPS)-stimulated murine macrophages was determined using previously described methods (Sang et al., 2004). In brief, to determine arachidonic acid release, RAW264.7 cells were incubated overnight with 0.1 μCi/ml [5,6,8,9,11,12,14,15-3H](N)-arachidonic acid to allow membrane incorporation. Cells were then washed with phosphate-buffered saline containing 0.1% bovine serum albumin. Cells were then stimulated with 2 μg/ml LPS for 1 h; the cells were washed; and fresh medium containing AcEGCG or EGCG (0–40 μM) was added. Following 18 h of incubation, radioactivity in the medium was determined by scintillation counting. To determine inhibition of NO formation, cells were stimulated and treated with test compounds as above. NO levels were determined by measuring nitrite production spectrophotometrically using the Greiss reagent (Ryu et al., 2003).

**Esterase-Mediated Deacytlation.** To determine the capability of plasma esterases to convert AcEGCG to EGCG, freshly isolated plasma from CF-1 mice was combined with AcEGCG (final concentration, 5 μM) and incubated at 37°C for 0 to 60 min. The reaction was stopped by addition of CH2Cl2, and the reaction time was held constant at 15 min. Samples were then prepared and analyzed as above.

**Oral Bioavailability.** Male, CF-1 mice (20–25 g, six per group) were given
Fig. 2. Biological activities and cellular uptake of AcEGCG. A, growth inhibition of KYSE150 human esophageal cancer cells and HCT116 human colon adenocarcinoma cells following treatment with either EGCG or AcEGCG for 24 h. Inhibition of NO production (B) or arachidonic acid release (C) by LPS-stimulated RAW264.7 murine macrophages by AcEGCG or EGCG (0–40 μM, 18-h treatment). D, cytosolic levels of EGCG in HCT116 human colon cancer cells treated with EGCG or AcEGCG (10 μM) for 0.25 to 24 h. n = 4 ± S.D. *p < 0.05; **p < 0.01.

Results

Synthesis and HPLC Analysis of AcEGCG. AcEGCG was obtained in 95% yield following peracetylation by reaction with acetic anhydride in a pyridine-catalyzed reaction. The structure was confirmed by one- (1H and 13C) and two-dimensional (HMBC and HMQC) NMR. 1H NMR [(CD3)2CO, 600 MHz]: δ 5.53 (1H, s, H-2), 7.57 (1H, m, H-3), 3.22 (1H, dd, J = 4.8, 18.0 Hz, H-4a), 3.11 (1H, dd, J = 1.8, 18.0 Hz, H-4b), 6.61 (1H, d, J = 2.4 Hz, H-6), 6.74 (1H, d, J = 2.4 Hz, H-8), 7.42 (2H, s, H-2' and H-6'), 7.63 (2H, s, H-2'' and H-6''), 2.29 (3H, CH3CO), 2.28 (6H, CH3CO), 2.28 (6H, CH3CO); 13C NMR [(CD3)2CO, 150 MHz]: δ 79.0 (d, C-2), 71.1 (d, C-3), 152.7 (s, C-4), 115.8 (d, C-5), 152.6 (s, C-6), 152.6 (s, C-7), 110.3 (d, C-8), 157.5 (s, C-9), 112.6 (s, C-10), 130.1 (s, C-1'), 121.6 (d, C-2' and C-6'), 146.4 (s, C-3' and C-5'), 137.3 (s, C-4'), 141.9 (s, C-1''), 124.5 (d, C-2'' and C-6''), 146.3 (s, C-3'' and C-5''), 138.4 (s, C-4''), 166.0 (s, C-7'), 22.6 (q, CH3CO), 22.3 (q, CH3CO), 22.2 (q, 2 × CH3CO), 22.1 (q, 2 × CH3CO), 21.7 (q, CH3CO), 21.6 (q, CH3CO), 171.1 (s, CH3CO), 170.6 (s, CH3CO), 170.0 (s, 2 × CH3CO), 169.9 (s, 2 × CH3CO), 169.1 (s, CH3CO), and 168.8 (s, CH3CO).

AcEGCG was not detectable using our standard electrochemical method for EGCG (Lee et al., 2002; Hong et al., 2003). Therefore, we developed an HPLC-UV method to analyze AcEGCG in cell culture medium and enzyme incubations. Analysis of standard solutions of AcEGCG revealed a single peak with a retention time of 12 min with a single dose of EGCG or AcEGCG (163.8 μmol/kg i.g.) and sacrificed at 20, 50, 90, 180, and 300 min. Plasma, small intestine, and colon were collected and processed by previously reported methods (Lee et al., 2002; Lambert et al., 2003). In brief, 100 μl of plasma was hydrolyzed with 1 U of sulfatase and 250 U of β-glucuronidase at 37°C for 45 min and extracted with 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 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.

HPLC Analysis of AcEGCG and EGCG. HPLC analysis of EGCG in cell culture medium and biological samples was performed using our previously reported methods (Lee et al., 2002; Hong et al., 2003). AcEGCG concentrations in enzyme reactions were analyzed by HPLC-UV with λmax = 280 nm. The mobile phase was a binary gradient of solvent A (4% sodium monophosphate buffer containing 5% acetonitrile, pH 3.2) and solvent B (1% sodium monophosphate buffer containing 70% acetonitrile, pH 3.2). The initial phase was a 12-min gradient from 57 to 86% B, followed by a 6-min isocratic period at 86% B. The mobile phase was then re-equilibrated at 57% B for 7 min. The flow rate was 1.5 ml/min. The limit of detection for the method was determined as the minimum quantifiable peak at a signal-to-noise ratio of 3 to 1.

Statistical Analyses. Pharmacokinetic parameters were calculated using Microsoft (Redmond, WA) Excel functions developed by Usansky and colleagues (http://www.boomer.org/pokin/soft.html). The area under the curve from 0 to infinity (AUC0-infinity) was calculated by the linear trapezoidal rule; elimination rate constant (k elim) was calculated from least-squares curve fit of the plot of ln concentration as a function of time; and the half-life (t1/2) was determined as ln(2)/k elim. Km and Vmax values for the de-esterification of AcEGCG were determined using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Differences in pharmacokinetic parameters, inhibition values, and cytosolic levels were assessed using the paired Student’s t test, and p < 0.05 was regarded as significant.
a $\lambda_{\text{max}} = 280$ nm. The apparent limit of detection was 400 ng/ml (Fig. 1). Although this limit of detection was not sensitive enough to detect AcEGCG levels in plasma and other biological samples, it was sufficient to monitor enzyme assays and cell uptake studies.

**Inhibition of Cell Growth, NO Production, and Arachidonic Acid Release.** The growth inhibitory activity of AcEGCG and EGCG were compared in KYSE150 human esophageal squamous cell carcinoma and HCT116 human colon adenocarcinoma cells (Fig. 2A). The IC$_{50}$ values of AcEGCG were approximately 10 and 32 µM against KYSE150 and HCT116 cells, respectively. These values were lower than those of EGCG (approximately 20 and 45 µM against KYSE150 and HCT116 cells) under similar treatment conditions.

The ability of AcEGCG and EGCG to inhibit NO production and arachidonic acid release by LPS-stimulated RAW 264.7 murine macrophages was compared. At 40 µM, AcEGCG inhibited NO production by 81%, whereas EGCG treatment reduced NO by only 15% (Fig. 2B). Similarly, AcEGCG reduced LPS-induced arachidonic acid release dose-dependently and was approximately 2-fold more potent than EGCG (Fig. 2C).

To determine whether the increased biological potency of AcEGCG was caused by increased cytosolic levels of EGCG in treated cells, we determined the cytosolic levels of EGCG in AcEGCG-treated HCT116 cells. Cells treated with AcEGCG had a maximal cytosolic concentration of 2088.0 ng/mg EGCG 2 h after treatment, whereas those treated with EGCG had a maximal cytosolic level of 65.0 ng/mg 6 h after treatment (Fig. 2D). After 24 h, the cytosolic levels of EGCG were 2.3-fold higher in cells treated with AcEGCG than in those treated with EGCG.

**Enzyme-Mediated Deacetylation of AcEGCG.** EGCG could be detected within 5 min following incubation of AcEGCG in mouse plasma (Fig. 3A). Time-dependent incubation with lower concentrations of mouse plasma and with hepatic microsomes resulted in decreases in the levels of AcEGCG and the formation of at least two putative metabolites (Fig. 3, B and C). We are currently trying to identify the structures of these compounds. Incubations in the absence of plasma or hepatic microsomes did not result in a significant decrease in the concentration of AcEGCG, nor the formation of M4.8 and M7.01 (data not shown). The kinetics of de-esterification of AcEGCG were determined in mouse plasma (Fig. 3D). Following a 10-min incubation, the $V_{\text{max}}$ and $K_{\text{m}}$ of de-esterification were determined as 10,615 pmol/min/mg and 16.7 µM, respectively.

**Oral Bioavailability of AcEGCG and EGCG.** The oral bioavailability of EGCG in CF-1 mice was compared following administration of equimolar doses of AcEGCG and EGCG. The peak concentration ($C_{\text{max}}$) of total EGCG in the plasma, small intestine, and colon was not significantly different between the two treatment groups (Fig. 4). By contrast, the AUC$_{0-\infty}$ of total EGCG increased by 2.4-, 2.8-, and 2.4-fold in the plasma, small intestine, and colon, respectively, of mice treated with AcEGCG compared with those treated with EGCG (Table 1). Likewise, the $t_{1/2}$ of total EGCG was increased by 2.2-, 2.4-, and 6.0-fold in the plasma, small intestine, and colon, respectively, in mice treated with AcEGCG compared with those treated with EGCG (Table 1). There was no significant difference in the lung levels of EGCG of the two treatment groups (data not shown).

**Discussion**

EGCG is the most abundant catechin in green tea (C. sinensis, Theaceae). This compound has been reported to have cancer-preventive activity in vivo and associated activities in vitro; however, it is extensively biotransformed and has relatively poor bioavailability (Lambert and Yang, 2003a,b). In an effort to improve the bioavailability of EGCG, we prepared AcEGCG and investigated the effect of peracetylation on the in vitro biological activities of EGCG related to cancer prevention and the bioavailability of AcEGCG in vivo.

Acetylation improved the cell uptake and growth inhibitory activity of EGCG against both human esophageal squamous cell carcinoma cells (KYSE150) and human colon adenocarcinoma cells (HCT116). Incubation of AcEGCG with KYSE150 cells resulted in rapid con-
version to EGCG and in the formation of at least two unknown compounds. These unknown compounds may represent partially deacetylated AcEGCG products. We are attempting to identify these putative metabolites. It appears that AcEGCG can increase the intracellular concentrations as a result of increased cell uptake over the short term (1 h), but once the compound is converted back to EGCG, it is then subject to biotransformation and efflux at a rate similar to EGCG over the long term (24 h). This may explain why the increase in growth inhibitory activity, which is measured after 24 h of treatment, was only a 2-fold. Based on these data, it would appear that AcEGCG in combination with inhibitors of phase II metabolism and active efflux would result in an even greater increase in biological potency.

AcEGCG increased the oral bioavailability of EGCG, as measured by AUC$_{0-\infty}$. Increases in the AUC$_{0-\infty}$ and t$_{1/2}$ of plasma (240 and 220%), small intestine (280 and 240%), and colon (240 and 600%) were observed following oral gavage with AcEGCG compared with treatment with equimolar doses of EGCG. Interestingly, there was little difference in the C$_{max}$ of EGCG between the AcEGCG- and EGCG-treated groups. This could be because of incomplete conversion of AcEGCG to EGCG following p.o. administration to mice. It is also possible that although acetylation is expected to increase absorption of the test compound, it is not expected to affect biotransformation and elimination of the deacetylated product, EGCG.

Because of limitations in the sensitivity of our current analytical method, we were unable to measure AcEGCG or partially deacetylated AcEGCG in the tissues of treated mice. Although we showed that AcEGCG is readily deacetylated by mouse plasma and hepatic microsomes, it is possible that a pool of partially deacetylated products is present in the tissue and plasma and that these products may have biological activity. We are currently developing a more sensitive method to study these partially deacetylated AcEGCG products.

In summary, we have shown that AcEGCG has improved biological activity in vitro and improved bioavailability in vivo. This compound is apparently readily converted back to EGCG both in vitro and in vivo, and the improved biological activity in vitro appears to be caused by increased intracellular levels of EGCG. These data suggest that AcEGCG may have improved cancer preventive activity in vivo. Future studies in animal models of carcinogenesis are needed to test this hypothesis.

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**References**


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