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Tea Catechin Auto-oxidation Dimers are Accumulated and Retained by Caco-2 Human Intestinal Cells

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Abstract

Despite the presence of bioactive catechin B-ring auto-oxidation dimers in tea, little is known regarding their absorption in humans. Our hypothesis for this research is that catechin auto-oxidation dimers are present in teas and are absorbable by human intestinal epithelial cells. Dimers [theasinensins (THSNs) and P-2 analogs] were quantified in commercial teas by HPLC-MS. (–)-Epigallocatechin (EGC) and (–)-epigallocatechin gallate (EGCG) homodimers were present at 10–43 and 0–62 $\mu\text{mol/g}$ leaf, respectively. EGC-EGCG heterodimers were present at 0–79 $\mu\text{mol/g}$. The potential intestinal absorption of these dimers was assessed using Caco-2 intestinal cells. Catechin monomers and dimers were detected in cells exposed to media containing monomers and preformed dimers. Accumulation of dimers was significantly greater than monomers from test media. Three h accumulation of EGC and EGCG was 0.19–0.55% and 1.24–1.35% respectively. Comparatively, 3h accumulation of the EGC P-2 analog, and THSNs C/E was $0.89 \pm 0.28\%$ and $1.53 \pm 0.36\%$. Accumulation of P-2, and THSNs A/D was $6.93 \pm 2.1\%$, and $10.1 \pm 3.6\%$. EGCG-EGC heterodimer P-2 analog, and THSN B 3h accumulation was $4.87 \pm 2.2\%$, and $4.65 \pm 2.8\%$ respectively. One h retention of P-2, and THSNs A/D was $171 \pm 22\%$, and $29.6 \pm 9.3\%$ of accumulated amount suggesting intracellular oxidative conversion of THSNs to P-2. These data suggest that catechin dimers present in the gut lumen may be readily absorbed by intestinal epithelium.

Keywords

catechins; epicatechins; auto-oxidation; tea; dimers; theasinensin; P-2; Caco-2

1. Introduction

Both epidemiological and experimental data have highlighted the potential disease preventative activities of tea catechins, including (–)-epigallocatechin gallate (EGCG) and (–)-epigallocatechin (EGC). These activities appear to include protection against oxidative stress and reduced risk of cardiovascular disease and certain cancers [1–7]. While the majority of studies have focused on monomeric catechins native to the tea leaf, *in vitro* experiments suggest

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that EGCG and EGC are extensively degraded at conditions near neutral pH, such as in cell culture media or during simulated digestion [8–14]. Under such conditions, EGCG forms several B-ring homodimers: theasinensins (THSNs) A/D (MW 914 g/mol, linked C₂–C₂ in the B-ring) and P-2 (884 g/mol, linked by B-ring opening/condensation) [8–11,14]. These B-ring dimers are structurally distinct from procyanidins and prodelphinidins, which are linked through the A/C-rings, and are found in apples, cocoa, and grapes. Recently, we characterized the oxidation of EGCG-EGC mixtures during *in vitro* digestion [15], demonstrating that EGC forms THSNs C/E (610 g/mol) and a P-2 analog (580 g/mol), and that EGCG and EGC combine to form THSN B and its unnamed isomers (762 g/mol) and a P-2 analog (732 g/mol) (Figure 1). While the extent to which these compounds may form in the gut or post-absorption is unknown [14], conditions favoring auto-oxidation do exist in the small intestinal lumen. This includes, elevated pH (≥ 6), residual dissolved O₂, presence of reactive oxygen species, and the absence of protection of cellular endogenous antioxidant systems [16–18].

These B-ring dimers are also intermediates generated during enzymatic oxidation (also referred to as fermentation) of catechins to form theaflavins (dimers with benzotropolone structures linked through the B-ring), and more complex products such as thearubigins, in oolong, and black teas originating from the leaves of *Camellia sinensis* [19–22]. Hashimoto *et al.* [22] demonstrated that THSNs A–G (THSNs F/G are dimers of ECG-EGCG) are components of oolong tea. Nonaka *et al.* [23] and Tanaka *et al.* [24] demonstrated the presence of THSNs A and B in mildly oxidized green tea (at 0.061% of total tea leaf weight and 0.5% of dry tea leaf weight, respectively). EGCG and EGC, predominant catechins present in green tea, are present at 4–12% and 2–6%, respectively, of the dry leaf weight [25–26]. Therefore, dimers may be present at up to 5–20% of the levels of the predominant monomers in mildly fermented green or oolong teas. Kusano *et al.* [27] also demonstrated the presence of THSNs A and B in black tea. These data suggest that the intake of THSNs and P-2 derivatives from mildly fermented teas (green, oolong and some black) may be appreciable. However, extensive quantitative profiling of these dimers in teas has yet to be performed.

In addition to their natural presence in teas and potential formation in the gut, studies have demonstrated appreciable biological activities for select catechin B-ring dimers. Yoshino *et al.* [9] reported that the THSNs and P-2 have Fe²⁺ chelation and O₂^{•−} scavenging activities equal to or greater than EGCG. Saeki *et al.* [28] demonstrated that THSN D was more effective than EGCG at inducing apoptosis in human histolytic lymphoma U937 cells, while P-2 had similar activity to EGCG. Pan *et al.* [29] further demonstrated that THSN A was more effective at inhibiting the growth of U937 cells than EGCG, and was also induced apoptosis. Maeda-Yamamoto *et al.* [30] suggest that THSN D was a better inhibitor of the invasive activity of fibrosarcoma HT1080 cells than EC and EGC, but was less effective than EGCG. Abe *et al.* [31] reported that THSN A was a potent inhibitor of rat squalene epoxidase, with an IC₅₀ (50% inhibitory concentration) value 5× lower than EGCG. Finally, Kusano *et al.* [27] demonstrated that THSN A was a more effective inhibitor of lipase than EGCG.

Despite the presence of catechin auto-oxidation dimers and their potential biological activities, little data exist regarding their absorption in humans, likely due to the fact that their dietary significance is not well established. Yoshino *et al.* [9] demonstrated that P-2 achieved 40% of the maximal plasma concentration of EGCG, and was eliminated more slowly, when orally administered to rats. A better understanding of the efficiency of absorption/accumulation in intestinal target tissues for THSN-type dimers and P-2 analogs compared to monomers is needed to extend our understanding of the role these compounds may play in chronic disease prevention.

Based upon these data, our hypothesis for this research was that catechin auto-oxidation dimers are relevant dietary compounds present in teas and are absorbable by human intestinal epithelial

cells. These dimers are likely in appreciable quantities, relative to monomeric catechins, and are therefore potentially relevant dietary components. The objectives of this study were to 1) quantify levels of catechin auto-oxidation dimers in a variety of commercial teas, 2) assess the absorption potential of dimers of EGCG and EGC using a Caco-2 human intestinal cell model, and 3) compare the efficiency of dimer absorption vs. monomers.

An understanding of the dietary levels as well as the potential intestinal absorption of THSNs and P-2 catechin derivatives will provide a framework for further investigation of the absorption and biological activities of these compounds in future animal and/or human feeding studies.

2. Methods and Materials

2.1 Tea Infusions

All teas were obtained from Upton Tea Imports (Hopkinton, MA) (Table 1). Hot water infusions were made in triplicate by brewing 2.2 g tea leaf in 250 mL boiling distilled/deionized water for 5 min with mild agitation. Infusions were then gravity-filtered through paper coffee filters to remove particulate matter. Aliquots of the filtrate (1 mL) were then diluted with 1 mL cold aqueous acetic acid (2% v/v), filtered through 0.45 μ m filters (National Scientific, Rockwood, TN), and analyzed by HPLC-MS.

2.2 Dimer Generation

Aqueous solutions of EGCG (0.25 mg/mL), ECG (0.25 mg/mL), and EGCG+ECG (0.25 mg/mL each) were prepared and divided into 2–15 mL aliquots (EGCG [teavigo®] was a gift from DSM Nutritional Products, Parsippany, NJ; EGC was obtained from Sigma-Aldrich, St. Louis, MO). One aliquot of each solution was acidified to pH 3 with 100 μ L 1 N HCl to minimize auto-oxidation. The second aliquot was adjusted to pH 7.2 with 0.1 N NaOH, blanketed with N₂, and incubated in a dark shaking water bath at 37°C for 2.5 h to favor pH-driven auto-oxidation reactions. Following incubation, oxidized solutions were acidified to pH 3 with 100 μ L 1 N HCl. For all solutions, 5 mL was stored at –80°C under N₂ prior to analysis. Media for cell accumulation experiments were prepared by diluting the remaining 10 mL of each solution (oxidized and non-oxidized) with 40 mL pH 5 phosphate-buffered saline (PBS: 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.14 M NaCl, 8.1 mM Na₂HPO₄, Invitrogen, Carlsbad, CA). Diluted dimer and monomer solutions were then filter-sterilized through 0.22 μ m Millipore Express PLUS membranes (Millipore Corp., Billerica, MA). Monomers/dimers in each solution were quantified in duplicate by extraction and HPLC analysis. Solutions (1 mL) were extracted 3 $\times$ with 3 mL ethyl acetate containing 4.5 mM butylated hydroxytoluene by vortexing, centrifugation (4500 rpm, 5 min, 4°C), and collection of the organic layer. The pooled extracts were dried under vacuum (37°C), dissolved in 200 μ L mobile phase A/phase B (90:10 v/v, see below) and analyzed by HPLC-MS.

2.3 Cell Culture

The TC7 clone of the Caco-2 human intestinal cell line was used as a model of intestinal absorption of catechin monomers and auto-oxidation B-ring dimers. Cells (passage 83–85) were seeded at a density of 6.4 $\times$ 10⁴ cells/well in 6-well polystyrene plates (9.5 cm²/well, Corning Inc., Corning, NY) and cultured to 10 d post-confluence to obtain highly-differentiated monolayers. Cells were cultured with complete Dulbecco's Modified Eagle's Medium (DMEM, Lonza Group, Basel, Switzerland) containing 10% (v/v) fetal bovine serum (FBS, JR Scientific Inc., Woodland, CA), with media changed every 2 d. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For accumulation experiments, monolayers were washed with PBS (pH 5), and 2.0 mL dilute dimer or monomer solutions/well was applied and incubated at 37°C for 0 min (removed immediately), 5 min, 15 min, 30 min, 1 h and 3 h (*n*=2

wells/trt for 0 min, $n=4$ wells/trt for 5 min–3 h). Monolayers were washed with 2 mL PBS (pH 5), washed with 2 mL 0.1% bovine albumin (w/v) in PBS (pH 5), covered with 1 mL cold PBS (pH 5), scraped from the plate, and stored at -80°C under N_2 prior to analysis. Protein values for cell monolayers were determined by the bicinchoninic acid (BCA) method using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), and were found to be 1.47 ± 0.03 mg/well. For extraction of native flavan-3-ols and auto-oxidation dimers, cells were thawed, sonicated for 15 s, and extracted as described above. The dried extracts were dissolved in 150 μL mobile phase A/phase B and analyzed by HPLC-MS.

2.4 Stability and Retention Experiments

In order to assess the stability of catechin monomers and dimers in the experimental media, cell-free control experiments were performed by incubating 2 mL aliquots of media 2 (oxidized EGCG) and media 5 (non-oxidized EGCG) under cell culture conditions (pH 5 PBS, 37°C) for 3 h in the absence of Caco-2 cells. Following incubation media was collected and analyzed by HPLC-MS for catechin monomer and dimer content.

The retention of accumulated monomers and dimers by Caco-2 cells was assessed in order to determine the potential for apical efflux of dimers relative to monomer catechins. Monolayers were exposed to 2 mL diluted (6-fold compared to accumulation experiments) test media 2 (oxidized EGCG) or media 5 (non-oxidized EGCG) for 3 h similar to the accumulation experiment. Following the 3 h accumulation period, half of the monolayers were collected for analysis and half were washed first with 2 mL PBS, and then incubated with a fresh 2 mL PBS for 1 h. Following incubation, monolayers were washed, collected, and analyzed as described previously for the accumulation experiment.

2.5 HPLC-MS Analysis

Tea infusions, dimer media and cell extracts were analyzed by reverse-phase (RP) HPLC with MS detection. Separations were performed on a Waters 2695 module (Milford, MA) with Waters XTerra RP- C_{18} guard and analytical columns (guard: 2.1×20 mm, $3.5 \mu\text{m}$ particle size; analytical column: 2.1×100 mm, $3.5 \mu\text{m}$ particle size) maintained at 40°C . Binary gradient elution was performed at 0.3 mL/min using the following mobile phases: A-0.4% formic acid in ddH_2O , B-4% isopropyl alcohol/0.4% formic acid in methanol. Separations were performed over 15 min using the following linear gradient: 85% A at 0 min, 30% A at 8 min, 20% A at 9 min, 0% A at 9.5 min, 85% A from 9.5 min to 15 min. Following separations, column effluent was split 1:1 for introduction by (–)-ESI into a Waters ZQ 2000 single quadrupole mass spectrometer. The ESI capillary and cone voltages were -3.5 kV and 35 V, respectively. The desolvation and source temperatures were 350 and 150°C , respectively. The desolvation and cone gas was N_2 at flow rates of 400 and 60 L/h, respectively. Monomers and dimers were detected by Selected Ion Response (SIR) monitoring of m/z values corresponding to deprotonated pseudomolecular ions ($[\text{M}-\text{H}]^{-}$) for catechin monomers [$m/z = 289, 305, 441$, and 457 for C/EC, GC/EGC, ECG, and EGCG, respectively] and dimers ($m/z = 579, 609, 731, 761, 883, 913$). SIR dwell times were 0.2 s for each m/z value, with inter-channel and interscan times of 0.01 s. SIR mass spans were ± 1 for each m/z value. For analysis of tea infusions, all SIRs were performed in one HPLC run.

Due to the lack of available B-ring dimer standards, response factors for catechins and catechin dimers were estimated by injecting equimolar concentrations (2.3 nmol to 19 pmol on-column) of EC and procyanidin B₂ [(–)-EC-(4 β →8)-(–)-EC] to obtain peak areas covering the range of the dimer peak areas. Preparation of standard curves of EC and procyanidin B₂ revealed that both curves exhibited two distinct linear regions with inflection points at similar concentrations. The relative response factors (i.e. slope ratios) of EC relative to procyanidin B₂ were 1.38 and 1.79 in the high and low peak area linear regions of the curves, respectively,

while the slopes were similar ($\pm 6\%$). Therefore, these response factors were used to construct standard curves for EGCG dimers and EGCG-EGC hetero-dimers from EGCG standard curves, and standard curves for EGC dimers from EGC standard curves, using the inflection points of the monomer curves to define the distinct linear regions of the estimated dimer.

As several isomers were present for each $[M-H]^-$ m/z value in media and cells, peaks of the same m/z value were quantified separately using standard curves, and the resulting concentrations were then summed to obtain a total concentration of isomers with the same $[M-H]^-$ m/z value. Differences in intracellular concentrations were determined by ANOVA using Fisher's protected least significant difference (Fisher's PLSD) test ($\alpha = 0.05$).

2.7 Statistical Analyses

Caco-2 experiments were designed to assess the potential for intestinal accumulation of catechin auto-oxidation dimers relative to their corresponding monomeric units. Experimental units included test media composition, incubation time (0–3h) and cellular retention. Based upon previous experiments in our laboratory examining the accumulation/retention of flavan-3-ol monomers by Caco-2 cell monolayers [32–34], a design using $n=4$ wells per treatment was selected having a calculated power of 0.95. Differences in intracellular catechin accumulation and retention at specific time points were determined by ANOVA using Fisher's protected least significant difference (Fisher's PLSD) test ($\alpha = 0.05$).

3. Results and Discussion

3.1 Quantitative Tea Profiles

Catechin monomers and B-ring dimers were detected in aqueous infusions of 16 high quality commercial teas by HPLC with (–)-ESI-MS detection. The predominant catechin monomers (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GC), EGC, (–)-epicatechin gallate (ECG), and EGCG were present at 3–34, 28–46, 2–9, 9–78, 42–121, 38–149 $\mu\text{mol/g}$ tea leaf, respectively (Table 1). Deprotonated pseudomolecular ions ($[M-H]^-$) corresponding to EGC homodimers, EGCG homodimers, and EGCG-EGC heterodimers were also detected at significant concentrations in the majority of teas (Table 1). EGC homodimers were present in all 16 teas, at concentrations of 11–29 and 10–43 $\mu\text{mol/g}$ tea leaf, for THSNs C/E and P-2 analogs, respectively. EGC homodimers were present at high concentrations in green, oolong, and black teas. This abundance in unfermented green teas reflects the extreme sensitivity of EGC to oxidative conditions [15]. EGCG homodimers were present at 0–13 and 0–62 $\mu\text{mol/g}$ tea leaf, for THSNs A/D and P-2 respectively, and were generally present at lower concentrations than EGC homodimers or EGCG-EGC heterodimers. EGCG homodimers were present at very low concentration in green teas (with P-2 detected in only 1 green tea), and their levels increased in oolong and black teas, corresponding to higher degrees of fermentation and oxidation. EGCGEGC heterodimers were present at 0–79 $\mu\text{mol/g}$ tea leaf, with the lowest levels in oolong teas. In general, black teas were the richest source of high levels of all dimer species. Representative chromatograms showing the profiles of EGC and EGCG homodimers in an aqueous infusion of Premium Formosa Oolong Choicest tea are presented in Figure 2. Comparisons of dimer concentrations with their precursor monomers in these teas indicate that the median levels of dimers in this survey of teas was 44–46 % of EGC levels for EGC homodimers, 1–4% of EGCG for EGCG homodimers, and 24–43% of EGC and 8–19% of EGCG for heterodimers, with extreme variation. In some cases, the dimers were present at greater levels (up to 800%) than the respective monomers. These findings are significant, as the levels of these dimers in commercially available teas and their corresponding brewed beverages have been largely unknown. These data suggest that THSNs and P-2 catechin derivatives are relevant dietary catechin forms that, in addition to catechin monomers and other components (theaflavins, thearubigins, etc.), might be biologically relevant constituents in

oolong and black fermented teas, as well as green teas (which typically undergo little or no fermentation).

3.2 Dimer Generation

Dimers for cell culture experiments were generated *in vitro* from authentic catechin standard material, as opposed to isolation from tea material, in order to more tightly control the profile of dimers of interest and eliminate other potentially interfering natural products (such as theaflavins) from cell culture experiments. This approach also allowed for generation of defined monomer/dimer ratios for subsequent studies. Oxidation of EGCG under experimental conditions (pH 7.2, 37°C for 2.5 h) resulted in generation of EGCG homodimers THSNs A/D (7.16% of added EGCG) and P-2 (10.8%). Oxidation of EGC resulted in the formation of EGC homodimers THSNs C/E (21.6% of added EGC) and the EGC dimer structurally analogous to P-2 (28.9%). The oxidation of the mixture of EGCG + EGC produced each of the EGCG and EGC homodimers, as well as the EGCG-EGC heterodimers THSNs B and its unnamed isomers (28.4% of EGCG, 32.6% of EGC) and the EGCG-EGC heterodimer structurally analogous to P-2 (17.0% of EGCG, 19.5% of EGC). These solutions (native and oxidized) were used to prepare test media for Caco-2 experiments. The HPLC-MS SIR profiles of compounds present in non-oxidized EGCG media (media 4) and oxidized EGCG media (media 2) are shown in Figure 3A–B. Similar representative profiles of dimers formed from EGC and EGCG + EGC treatments (monomers and dimers in oxidized/non-oxidized media) were observed (data not shown).

The concentrations of the compounds of interest in each media are described in Table 2. Epimers of EGCG (GCG) and EGC (GC) were also formed (data not shown) during oxidation, along with isomers with of each dimer. The formation of these epimers likely explains the fact that multiple isomers of each dimer were observed, as GCG and GC are also likely to undergo dimerization as previously observed [15]. As several stereochemical configurations of these compounds were detected, the concentrations listed in Table 2 represent the sum of all isomers for each specific $[M-H]^-$ m/z value in specified media. Catechin dimers were present in media 1–3 at roughly 4–16 μ M compared to 80–100 μ M for monomers in media 4–6. This ratio, roughly 4–20% of monomer concentrations, approximates the ratio previously observed in green tea [23–26].

3.3 Cell Culture Experiments

Following exposure of Caco-2 monolayers to monomer or dimer media for 0 to 3 h at physiologically relevant concentration ratios, monomers and selected isomers of each dimer were detected in the respective cell extracts. The HPLC-MS SIR profiles of compounds present in Caco-2 cells fed media 2 for 3 h, and cells fed catechin/dimer-free PBS are shown in Figure 3C–D. Similar representative profiles of EGC and EGCG + EGC treatments (monomers and dimers cells) were observed (data not shown). To confirm that dimers were in fact absorbed by Caco-2 cells and not exclusively adsorbed onto the apical membrane, additional MS-MS analysis of selected extracts were performed to detect known catechin phase-II metabolites previously formed by Caco-2 cells in culture [35–36]. Several dimer metabolites were detected, including an O-methylated derivative of the EGC P-2 analog ($[M-H]^- = 593$) and a sulfated conjugate of THSN C/E ($[M-H]^- = 689$), in agreement with previous data suggesting that O-methylated and sulfated derivatives are the predominant phase-II metabolites of catechins formed in Caco-2 cells [37]. MS-MS spectra of these metabolites are presented in Figures 4–5. These data further suggest that dimers present in test media were indeed absorbed and metabolized by Caco-2 cell monolayers. The extent of metabolism of these catechin oxidation products, as well as the position of Phase-II conjugation, merits further investigation.

The net accumulation of dimers and monomers was calculated as the percentage of the total amount of each compound in the respective test media (all isomers at the specified m/z value) that was accumulated in cells (2 mL media/well) at the specified time point. The percentage accumulation of dimers and monomers over 3 h is shown in Figure 6. The net accumulation at 3 h of EGC, the EGC P-2 analog (m/z 579), and THSNs C/E was $0.55 \pm 0.07\%$ (0.60 ± 0.08 nmol/mg), $0.89 \pm 0.28\%$ (0.14 ± 0.04 nmol/mg), and $1.53 \pm 0.36\%$ (0.18 ± 0.04 nmol/mg), respectively. The net accumulation at 3 h of EGCG, P-2, and THSNs A/D was $1.35 \pm 0.16\%$ (1.95 ± 0.21 nmol/mg), $6.93 \pm 2.1\%$ (0.54 ± 0.16 nmol/mg), and $10.1 \pm 3.6\%$ (0.52 ± 0.18 nmol/mg), respectively. The net accumulation at 3 h of EGCG, EGC, the heterodimer P-2 analog, and THSNs B (and its unnamed isomers) was $1.24 \pm 0.10\%$ (1.89 ± 0.15 nmol/mg), $0.19 \pm 0.02\%$ (0.25 ± 0.03 nmol/mg), $4.87 \pm 2.2\%$ (0.63 ± 0.28 nmol/mg), $4.65 \pm 2.8\%$ (1.00 ± 0.60 nmol/mg), respectively. These data demonstrate that catechin B-ring dimers have a significantly greater ($P < 0.05$) net accumulation (on a % basis) than the monomers, albeit with greater variability. These findings appear to agree with the results of Deprez *et al.* [38], who reported that procyanidin dimer B3 [(+)-C-(4 α →8)-(+)-C] had an apparent apical → basolateral permeability value in Caco-2 monolayers twice that of (+)-C (2.0×10^{-6} and 0.8×10^{-6} cm/s, respectively).

The accumulation of monomers over 3 h agrees with our previously reported result that 3 h accumulation of EGCG and EGC was 0.2–2.5 and 0.1–0.5 nmol/mg protein, respectively [32]. Additionally, our results agree with those of Vaidyanathan and Walle [39], who reported the 1 h accumulation of EGC from media containing 50 μ M EGC to be roughly 3 nmol/mg protein. The higher accumulation of EGCG relative to EGC, in spite of the higher reported apical to basolateral permeability of EGC, is likely explained by the greater apical efflux of EGC back to the media [37]. As apparent from Figure 6, EGCG was accumulated at appreciable amounts even at 0 min, but neither EGC nor any of the dimers exhibited this phenomenon. This result may be due to adsorption of EGCG on to the apical membrane, and is supported by previous studies demonstrating this behavior for EGC and other highly hydroxylated flavonoids [39–40].

Between catechin monomers and their respective dimers, statistically significant differences in accumulation at 3 h were found between EGCG and THSNs A/D (m/z 913) and between EGC and THSNs C/E (m/z 609). No statistically significant differences in accumulation were found between EGCG or EGC and their heterodimers. All other differences between monomers and their auto-oxidation products were not significant, due in large part to the high variation in the dimer accumulation at the 3h time point. The larger variation in the dimer accumulation data relative to the monomers could be due to several factors. First, the larger accumulation means would be expected to have larger relative SEM values. However, examination of the data suggests that the % CV values were larger for dimer accumulation values (47–120%) than for the monomer accumulation values (16–26%) at 3 h. This suggests that the higher variability in accumulation may be due to differences in stability of dimers compared to the monomers in the experimental media, or potential differences in the stability or metabolism of dimers compared to monomers inside the cell.

In order to assess the stability of catechin monomers and dimers in the experimental media, cell-free controls were performed by incubating 2 mL aliquots of media 2 (oxidized EGCG) and media 5 (non-oxidized EGCG) under cell culture conditions (37°C) for 3 h. The stability of EGCG, P-2, and the THSNs over 3 h were 91.8 ± 6.4 , 94.6 ± 1.0 , and $100 \pm 0.36\%$, respectively (mean $\pm$ SEM for $n = 4$ replicates, see Figure 7A). This suggests high stability of both monomers and dimers in media (pH 5), and that the higher variability of dimer accumulation may be a result of processes occurring during or post-absorption by Caco-2 monolayers, including differences in rate and extent of metabolism or further oxidative degradation of EGCG to dimers and THSNs to form P-2 intracellularly.

The apparent increase in accumulation of auto-oxidation dimers relative to monomers may be a result of several factors. First, accumulation in the current studies represents the net sum of absorption, surface adsorption, melting into the apical membrane, and apical efflux of the compounds of interest. Additionally, potential instability (conversion of monomers → dimers and dimers → other products) intracellularly could alter the profile of absorbed compounds and thus influence the observed intracellular accumulation as assessed at any one timepoint. To minimize the influence of surface adsorption on accumulation data, cells were washed with 0.1% bovine albumin before collection to remove any monomers or dimers plated onto the apical membrane. Additionally, the membrane adsorption of (+)-C and procyanidin B3 was previously demonstrated by Deprez *et al.* [38] to be similar (roughly 0.15–0.2 % transmembrane resistance, $\Omega \text{ cm}^2$). Therefore, the contribution of membrane adsorption is not likely to have resulted in the differences in measured cellular accumulation observed between monomers and dimers.

In order to further investigate potential post-absorption differences [degradation of dimers and/or apical efflux by xenobiotic ABC transporters such as P-glycoprotein (Pgp) and multidrug resistance protein (MRP2)] [39,41–43], a parallel accumulation and retention experiment was subsequently performed. Cells were exposed to 2 mL diluted media 2 (oxidized EGCG) or media 5 (non-oxidized EGCG) for 3 h similar to the accumulation experiment. Following the 3 h accumulation period, half of the monolayers were collected for analysis and half were then incubated 2 mL PBS for 1 h. The results of retention experiments are shown in Figure 7B and Table 3. One h retention of EGCG, P-2 (m/z 883 isomers), and THSNs A/D (m/z 913 isomers) was 56.8 ± 0.81 , 171 ± 22 , and $29.6 \pm 9.3\%$, respectively, of the amount present in cells following 3 h of accumulation. Statistically significant differences in retention were observed for P-2 vs. EGCG and THSNs A/D.

These data suggest the potential conversion of THSNs to form P-2 following absorption, as the THSNs were highly accumulated in the presence of media (Figure 6) but poorly retained in the absence of media. This conversion is most likely occurring intracellularly, due to the fact that 1) the retention of P-2 was $> 100\%$, 2) the stability of THSNs in cell free media was $100 \pm 0.36\%$, and 3) the THSNs are unlikely to have been effluxed to a greater extent than EGCG, which shares similar structure but is half the size of the THSNs. Additionally, THSNs are not stable end-products of EGCG auto-oxidations, as they have been shown to undergo further oxidation [8,10–11,14]. Several studies have shown that THSN A/D degradation is accompanied by the generation of P-2, and that P-2 is more stable than THSN A/D, suggesting that THSN A/D are intermediate oxidation products between EGCG and P-2 [8–11]. Therefore, once absorbed, intracellular conditions may provide an environment suitable for further oxidative degradation of THSN A/D to form P-2, resulting in higher cellular accumulation of this specific catechin auto-oxidation product over time. In light of these data, additional controlled experiments are warranted to elucidate the specific metabolism and reactivity of the THSNs in the cell interior and specific bioactivities of both THSNs and P-2 catechin derivatives.

Based on our profiling of tea infusions (Table 1), we accept our hypothesis that catechin auto-oxidation dimers are in fact present in a wide variety of commercially-available teas. Furthermore, observed Caco-2 accumulation data supports our hypothesis that catechin auto-oxidation dimers are absorbable by human intestinal epithelial cells, in appreciable quantities relative to monomeric catechins. Combined these data support the notion that catechin auto-oxidation products formed through processing are potentially relevant dietary polyphenol forms that merit further study.

Preformed homo- and heterodimers formed during oxidation of monomeric catechins appear to be readily absorbed and well retained by Caco-2 monolayers. Additionally, THSNs A/D

appear to be degraded to form P-2 upon absorption by intestinal cells. Combined, these data further suggest that the observed differences in the bioavailability of EGCG and EGCG auto-oxidation dimers may be due in part to differences in intestinal uptake as well as efflux of dimers such as P-2 in the intestine. These findings, using a relevant human intestinal cell culture model, provide preliminary insight into the absorptive potential of these unique dietary polyphenol forms. In vivo perfusion experiments in rodents have previously demonstrated the trans-epithelial transport of similar catechin dimers (procyanidins) in the intestine is extremely low compared to the precursor monomer [44]. Combined with the current study, this suggests that the intestinal epithelia may selectively accumulate relatively higher levels of these dimers if present in the diet, or if formed through digestion of monomeric catechins. Therefore, the intestinal epithelia may be relevant tissue to investigate specific biological effects of these dimers, and systemic (i.e. blood) bioavailability studies may not adequately characterize the biological relevance and distribution of these compounds. In light of the potential consumption of catechin B-ring dimers from brewed tea and their possible formation during digestion, as well as their apparent absorption and accumulation by intestinal cells, catechin B-ring dimers merit further investigation as potentially bioavailable and bioactive agents in humans.

It is important to note that a limitation of the present study is the use of Caco-2 monolayers in a single compartment model to assess absorption and retention. While providing valuable information on accumulation of these polyphenol forms by intestinal cells, this model provides only preliminary information on the potential for systemic bioavailability, and metabolism, of these auto-oxidation products. Future studies using Caco-2 may be designed employing transwell inserts to facilitate observation of trans-cellular (apical → basolateral) transport of dimers. Eventually, physiological relevance of these catechin-derived compounds must be established using relevant *in vivo* models.

Abbreviations

C	(+)-catechin
CV	coefficient of variation
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
GC	(-)-gallocatechin
IC ₅₀	50% inhibitory concentration
[M-H] ⁻	deprotonated pseudomolecular ion
MRP	multidrug resistance protein
Pgp	p-glycoprotein
PTFE	poly(tetrafluoroethylene)
SEM	standard error of the mean
SIR	selected Ion Response
THSNs	theasinensins and isomers

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Linkage	Type	Compound	Precursors	MW ^a (g/mol)	Substitution ^b	
					R ₁	R ₁ '
C2'-C2'	Homo	THSN A/D	2 EGCG	914	G	G
	Homo	THSN C/E	2 EGC	610	OH	OH
	Hetero	THSNB	EGCG + EGC	762	G	OH
B-ring opening	Homo	P-2	2 EGCG	884	G	G
	Homo	P-2 analog	2 EGC	580	OH	OH
	Hetero	P-2 analog	EGCG + EGC	732	G/OH	OH/G

^aNominal molecular weight

^bG = galloyl residue

Figure 1.

Structures of EGCG and EGC, as well as the known auto-oxidation dimers (THSNs and P-2 analogs) of EGCG and EGC formed through *in vitro* digestion, incubation in a variety of fluids at near-neutral pH (cell culture media, authentic intestinal juices, plasma, etc.), and enzymatic oxidation both *in vitro* as well as in tea.

Figure 2.

HPLC-MS Selected Ion Response (SIR) chromatograms representing the profile of EGC and EGCG ($[M-H]^- = 305$ and 457 , respectively) and EGC- and EGCG-derived auto-oxidation homodimers ($[M-H]^- = m/z$ 579, 609 for EGC homodimers; $[M-H]^- = m/z$ 883, 913 for EGCG homodimers) present in an aqueous infusion of Premium Formosa Oolong Choicest (Upton, Hopkinton, MA) tea leaves. SIR chromatograms are scaled to the highest peak in each trace.

Experimental. Aqueous tea infusion were made by brewing 2.2 g tea leaf in 250 mL boiling distilled/deionized water for 5 min with mild agitation. Aliquots of infusions were then diluted (1:1) with cold aqueous acetic acid (2% v/v) and analyzed by HPLC-MS. Monomers and dimers

were detected by SIR monitoring of m/z values corresponding to deprotonated pseudomolecular ions ($[M-H]^-$) for catechin monomers and dimers.

Figure 3.

HPLC-MS SIR chromatograms representing the profile of EGCG ([M-H]⁻ = m/z 457) and EGCG-derived auto-oxidation dimers (EGCG-derived THSNs [M-H]⁻ = m/z 913, P-2 [M-H]⁻ = m/z 883) present in concentrated extracts of media 5 (**A**, non-oxidized EGCG) and media 2 (**B**, oxidized EGCG) as well as the cell extracts obtained following exposure of Caco-2 cells to media 2 (**C**) or to catechin/dimer-free PBS (**D**) for 3 h. **A**, **B**, and **C** are scaled to the highest peak in each set of chromatograms, and **D** is scaled to **C**. **Experimental.** Non-oxidized media was prepared from EGCG (0.25 mg/mL). Oxidized media was prepared from EGCG (0.25 mg/mL) incubated at pH 7.2, 37°C for 2.5 h to drive auto-oxidation reactions. Media for cell accumulation experiments were prepared by diluting oxidized and non-oxidized solutions

5-fold with pH 5 PBS. Monomers/dimers in each solution were identified by extraction with ethyl acetate/4.5 mM BHT and HPLC-MS analysis. For cell experiments, differentiated monolayers of Caco-2 human intestinal cells were employed. For accumulation experiments, dilute dimer media (oxidized), monomer media (non-oxidized), or PBS solution was applied (2 ml/well) and incubated at 37°C for 3 h. Monolayers were washed with PBS (pH 5), 0.1% bovine albumin in PBS (pH 5) and scraped in pH 5 PBS. For extraction of native flavan-3-ols and auto-oxidation dimers, cells were extracted as for media. Media and cell extracts were analyzed by HPLC-MS.

Figure 4.

MS-MS spectra of an EGC homodimer (THSN C/E) in an oxidized EGC solution (**top**) and a compound tentatively identified as the O-methylated form of the same compound, observed in Caco-2 cells fed oxidized EGC containing THSNs C/E (**bottom**). **Experimental.** See Figure 3 for media, cell culture, and extraction conditions. For identification of dimers in media and cells, extracts of both were analyzed by HPLC-MS/MS. Spectra were obtained by (–)-ESI-MS-MS analysis on a Waters Q-TOF Micromass spectrometer. ESI capillary, cone, and extraction voltages were –3.5 kV, 40 V, and 1 V, respectively. The source and desolvation temperatures were 150 and 300°C, respectively. The nebulizer cone and desolvation gasses were N₂ at flow rates of 50 and 500 L/hr, respectively. Quadrupole pre- and post-filter voltages,

ion quadrupole energy, and radio frequency/direct current offset voltages were 5.0 V, 5.0 V, 0.5 and 3.0 V, respectively. For MS-MS analysis, the pseudomolecular ions of interest ($[M-H]^-$ $m/z = 579$ for the dimer 593 for the O-methylated dimer) were fragmented by collision-activated dissociation (CAD). The collision target gas was Ar (69 kPa), and ion kinetic collision energies were 20–30 eV. Mass data was collected from m/z 50–1500, with scan time interscan delay of 0.5 s and 0.1 s, respectively. Adapted from [15].

Figure 5.

MS-MS spectra of an EGC homodimer (P-2 analog) in an oxidized EGC solution (**top**) and a compound tentatively identified as the sulfated conjugate of the same compound, observed in Caco-2 cells fed oxidized EGC containing the P-2 analog (**bottom**). **Experimental.** Refer to Figures 3 and 4 for experimental conditions. For MS-MS analysis, the pseudomolecular ions of interest were $[M-H]^-$ $m/z = 609$ (dimers) and 689 (sulfated dimer), respectively. Adapted from [15].

Figure 6.

Percent accumulation of EGC monomers (A) and dimers (B), EGCG monomers (C) and dimers (D), and EGCG-EGC monomers (E) and dimers (F) by Caco-2 cells. Percent accumulation represents the percent of the total amount of each compound in the respective media that was accumulated in cells (2 mL media/well) at the specified time point. Error bars represent mean $\pm$ SEM from $n=2$ (0 min) or $n=4$ (5 min–3 h) replicate wells. Significant differences between accumulation of dimers and the respective monomer precursors at the same time point are indicated by * ($P < 0.05$, by ANOVA using Fisher's PLSD). **Experimental.** Cells (TC7 clone of Caco-2 human intestinal cell line, passage 83–85) were seeded and cultured as described in *Methods and Materials*. For accumulation experiments, Caco-2 monolayers were washed with

PBS (pH 5), and dilute dimer or monomer solutions (2 mL/well) was applied and incubated at 37°C for 0 min-3 h ($n=2$ wells/trt for 0 min, $n=4$ wells/trt for 5 min-3 h). Monolayers were washed with pH 5 PBS, 0.1% bovine albumin in pH 5 PBS, and collected in pH 5 PBS. For extraction of native flavan-3-ols and auto-oxidation dimers, cells were extracted with ethyl acetate/4.5 mM BHT. The dried extracts were dissolved in 150 μ L and analyzed by HPLC-MS.

Figure 7.

(**A**) Stability (expressed as a percent of initial concentrations) of EGCG (media 5) and EGCG dimers (m/z 883 and 913, media 2) during incubation in cell-free loading media (see Figure 3) in 6-well plates for 3 h under cell culture conditions. (**B**) Percent retention of EGCG (media 5) and EGCG dimers (m/z 883 and 913, media 2) by Caco-2 cells. **Experimental.** Percent retention represents the percent of the total amount of each compound in the cells following exposure to 2 mL of the respective media for 3 h that remained in the cells following removal of media and incubation with 2 mL catechin/dimer-free PBS for 1 h. Quantification was performed by HPLC-MS as described in Figure 2. Data represent mean $\pm$ SEM from $n=4$

replicates. Presence of different superscripts indicates a significant difference in retention ($P < 0.05$, by ANOVA using Fisher's PLSD).

Table 1

Concentrations of catechin monomers and dimers in assorted green, black, and oolong teas^a

Compound	Monomers					Dimers					THSN A/D
	C	EC	GC	EGC	ECG	EGCG	P-2 analog	THSN C/E	P-2 analog	THSN B ^b	
[M-H] ⁻ (m/z)	289	289	305	305	441	457	579	609	731	761	913
Concentration in Leaf ^d (μmol/g)											
Green Teas											
Darjeeling Green Tea	28.6 ± 0.058	34.3 ± 0.088	8.49 ± 0.43	51.0 ± 1.3	91.7 ± 0.71	137 ± 1.4	20.6 ± 0.29	14.2 ± 0.14	10.6 ± 0.12	26.8 ± 0.20	ND
Thailand Bold Leaf Green Tea	34.0 ± 0.17	46.2 ± 0.29	6.51 ± 0.432	23.3 ± 0.51	121 ± 2.4	56.2 ± 0.96	25.3 ± 0.63	43.1 ± 0.33	55.5 ± 0.73	ND	ND
Japanese Green First Grade Bancha	10.9 ± 0.31	37.4 ± 0.14	7.12 ± 0.0040	69.7 ± 0.64	81.7 ± 1.1	126 ± 2.14	23.1 ± 0.27	19.0 ± 0.16	8.77 ± 0.32	23.4 ± 1.1	ND
Vietnam Green Sencha	28.1 ± 0.029	37.2 ± 0.50	8.77 ± 0.54	70.1 ± 2.4	75.0 ± 1.4	109 ± 1.7	21.2 ± 0.31	34.1 ± 0.52	18.7 ± 0.91	48.5 ± 0.14	ND
Japanese Gyokuro	10.0 ± 0.23	38.0 ± 0.44	6.45 ± 0.34	77.6 ± 1.4	78.9 ± 2.1	149 ± 4.2	21.6 ± 0.044	24.1 ± 0.39	6.37 ± 0.31	8.73 ± 0.69	ND
China First Grade Gunpowder	6.97 ± 0.44	33.7 ± 0.28	4.84 ± 0.43	46.8 ± 1.1	66.4 ± 1.8	93.8 ± 2.8	21.6 ± 0.45	20.1 ± 0.34	5.82 ± 0.19	11.4 ± 0.49	2.47 ± 0.065
China GT Chun Mee Moon Palace	10.8 ± 0.48	36.3 ± 0.17	6.61 ± 0.32	66.2 ± 0.85	82.7 ± 1.5	142 ± 1.7	26.8 ± 0.21	18.4 ± 0.23	7.56 ± 0.27	16.2 ± 0.73	ND
Range	10-34	34-46	6.5-9	23-78	66-121	56-149	21-27	18-43	5.8-56	0-49	0-7.9
Black Teas											
Orthodox BOP Darjeeling	10.8 ± 0.25	31.5 ± 0.16	3.42 ± 0.036	17.3 ± 0.56	116 ± 1.1	126 ± 2.1	24.7 ± 0.31	33.1 ± 0.47	16.2 ± 0.86	79.4 ± 0.27	3.66 ± 0.075
Arya Estate Darjeeling	15.7 ± 4.7	29.8 ± 0.033	3.30 ± 0.163	8.84 ± 0.18	106 ± 1.7	77.5 ± 2.2	28.9 ± 0.67	34.8 ± 0.75	23.6 ± 1.2	72.7 ± 6.0	11.2 ± 0.25
Premium Blend Darjeeling	17.4 ± 5.3	31.0 ± 0.089	5.29 ± 0.52	16.0 ± 0.13	97.3 ± 1.1	87.8 ± 0.95	21.4 ± 0.32	29.5 ± 0.078	16.2 ± 0.29	66.9 ± 5.4	13.0 ± 0.365
Poobong Estate Darjeeling	9.73 ± 0.14	30.1 ± 0.059	5.22 ± 0.027	17.8 ± 0.048	86.9 ± 0.50	115 ± 0.88	18.9 ± 0.22	27.5 ± 0.071	8.43 ± 0.13	33.6 ± 0.77	7.34 ± 0.29
Range	9.7-17	30-32	3.4-5.3	8.8-18	87-116	78-126	19-29	28-35	8.4-24	34-79	3.7-13
Oolong Teas											
Premium Formosa Oolong Choicest	3.10 ± 0.13	28.2 ± 0.054	1.82 ± 0.068	13.2 ± 0.41	43.1 ± 0.37	38.3 ± 0.54	11.4 ± 0.35	11.8 ± 0.074	4.80 ± 0.21	12.2 ± 0.21	3.36 ± 0.11
Formosa Oolong Fine Grade	5.54 ± 0.14	29.5 ± 0.13	4.25 ± 0.13	19.8 ± 0.35	50.6 ± 0.22	52.1 ± 0.58	10.7 ± 0.20	25.2 ± 0.25	6.10 ± 0.32	25.8 ± 0.53	6.16 ± 0.16
Formosa Amber Oolong	4.74 ± 0.039	31.0 ± 0.12	4.65 ± 0.51	39.1 ± 0.76	46.2 ± 0.63	64.9 ± 0.37	14.1 ± 0.41	9.99 ± 0.20	4.50 ± 0.076	ND	3.66 ± 0.23
										ND	ND

Compound	Monomers					Dimers					THSN A/D
	C	EC	GC	EGC	EGCG	P-2 analog	THSN C/E	P-2 analog	THSN B ^b	P-2	
[M-H] ⁻ (m/z)	289	289	305	441	457	579	609	731	761	883	913
Concentration in Leaf ^d (μmol/g)											
Green Teas											
China Oolong Se Chung	3.87 ± 0.17	30.2 ± 0.12	4.14 ± 0.33	31.7 ± 0.85	42.1 ± 0.33	58.2 ± 0.97	13.1 ± 0.14	11.1 ± 0.14	ND	ND	ND
Formosa Jade Oolong	6.05 ± 0.30	31.5 ± 0.14	4.88 ± 0.29	41.4 ± 0.99	49.7 ± 0.62	75.5 ± 1.3	12.4 ± 0.28	10.1 ± 0.20	ND	ND	ND
Range	3.8-6.1	28-32	1.8-4.7	13-41	42-51	38-76	11-14	10-25	0-26	0-6.2	0-13

Experimental: All teas were obtained from Upton Tea Imports (Hopkinton, MA). Hot water infusions were made in triplicate by brewing 2.2 g tea leaf in 250 mL boiling distilled/deionized water for 5 min with mild agitation. Infusions were then gravity-filtered through paper coffee filters to remove particulate matter. Aliquots of the filtrate (1 mL) were then diluted with 1 mL cold aqueous acetic acid (2% v/v), filtered through 0.45 μm filters, and analyzed by HPLC-MS.

THSN B has unnamed isomers, similar to A/D, C/E, and F/G. These isomers are included in the values reported here

Concentrations reported as mean ± SEM from n=3 triplicate analyses, leaf weight represents weight as purchased

ND = not detected

Table 2

Concentrations of selected compounds of interest in media prepared from oxidized catechins (media 1–3) or native catechins (media 4–6) and fed to highly differentiated Caco-2 human intestinal cell monolayers.

Media ^a	Type	Compound	Precursors	MW (g/mol)	[M–H] [–] (m/z)	Concentration ^{b,c} (μM)	Conversion ^{d,e} (%)
1	oxidized	P-2 analog	2 EGC	580	579	11.6	28.9
		THSN C/E	2EGC	610	609	8.68	21.6
2	oxidized	P-2	2 EGCG	884	883	5.75	10.8
		THSN A/D	2 EGCG	914	913	3.81	7.16
3	oxidized	P-2 analog	EGCG + EGC	732	731	9.48	17.0, 19.5
		THSN B ^f	EGCG + EGC	762	761	15.9	28.4, 32.6
4	native	EGC	–	306	305	80.3	–
5	native	EGCG	–	458	457	106	–
6	native	EGC	–	306	305	97.5	–
		EGCG	–	458	457	112	–

^aMedia 1–3 were prepared from oxidized EGC (1), EGCG (2), or EGCG + EGC (3) (catechins oxidized at pH 7.2 for 2.5 h). Media 1–4 were prepared from native unoxidized EGC (4), EGCG (5), or EGCG + EGC (6).

^bConcentrations were determined by HPLC with (–)-ESI-MS by selected ion response (SIR) monitoring, and represent the sum of all isomers with SIR response at the specified pseudomolecular ion ([M–H][–]) m/z values. Concentration values represent the mean of n=2 replicate determinations.

^cCells were fed 2 mL media per well.

^dPercentage of added monomer residues converted to the specified dimer species during oxidation (each dimer contains 2 monomer residues)

^ePercent conversion values for m/z 731 and 762 are reported for EGCG, EGC

^fTHSN B has unnamed isomers, similar to A/D, C/E, and F/G. These isomers are included in the values reported here

Table 3

Accumulation and retention of EGCG and EGCG-derived auto-oxidation dimers by highly differentiated Caco-2 intestinal cell monolayers^{abc}.

		EGCG	P-2 (883 m/z)	THSN A/D (913 m/z)
Media^{de}	Concentration (μM)	17.7	0.958	0.635
3 h Accumulation	Absolute (pmol/well)	488 ± 33	28.6 ± 17	23.9 ± 10
	% of Fed	1.38 ± 0.092	1.50 ± 0.90	1.88 ± 0.80
1 h Retention	Absolute (pmol/well)	277 ± 3.9	48.9 ± 6.4	7.08 ± 2.2
	% of Accumulated	56.8 ± 0.81	171 ± 22	29.6 ± 9.3

^aExperimental: To assess stability of monomers and dimers in the experimental media, cell-free controls were performed by incubating media 2 (oxidized EGCG) and media 5 (unoxidized EGCG) under cell culture conditions (pH 5 PBS, 537°C) for 3 h in the absence of Caco-2 cells. Media were analyzed by HPLC-MS. To assess retention of accumulated monomers and dimers, cells were exposed to diluted (6-fold compared to accumulation experiments) media 2 (oxidized EGCG) or media 5 (unoxidized EGCG) for 3 h similar to the accumulation experiment. Following the 3 h accumulation period, half of the monolayers were collected for analysis and half were washed with PBS, washed with 0.1% bovine albumin in PBS, and then incubated PBS for 1 h. Monolayers were then washed, collected, and extracted with 3 mL ethyl acetate/4.5 mM BHT. The dried extracts were dissolved in 150 μL and analyzed by HPLC-MS.

^bAccumulation and retention values represent the mean ± SEM of *n*=4 replicates

^cCells for the retention experiment were cultured to 14d post-confluence

^dNote that media for retention experiment was diluted 6-fold compared to accumulation experimental media (compare with Table 1).

^eRefer to Table 1 for details of media preparation and quantification.