THE PENNSYLVANIA STATE UNIVERSITY
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THE SYNERGISTIC INHIBITION OF HUMAN LUNG CANCER CELLS BY THE TEA POLYPHENOL (-)-EPIGALLOCATECHIN-3-GALLATE AND CLINICALLY PRESCRIBED INHIBITORS OF CATECHOL-*O*-METHYLTRANSFERASE

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ABSTRACT

Tea, produced from the leaves of *Camellia sinensis,* was initially utilized for its medicinal properties nearly 5,000 years ago.[1] Current worldwide consumption of tea as a beverage is second only to that of water, with approximately three billion kilograms being processed and consumed annually. Tea contains several polyphenolic compounds known as catechins. These catechins have been studied extensively for their cancer chemopreventive properties both *in vitro* and *in vivo* within animal and human systems. (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin and has been implicated as the most bioactive.

Catechol-*O*-methyltransferase (COMT) is a phase II metabolizing enzyme that reduces the activity/toxicity of catechol structures through conjugation with methyl groups. EGCG is readily methylated by COMT in both rodents and humans. An increasing body of evidence suggests that methylation by COMT modulates the chemo-preventive activity of EGCG.

The purpose of the present study was to determine if EGCG in combination with clinically used COMT inhibitors (entacapone and tolcapone) has synergistic anticancer effects in the H1299 human lung adenocarcinoma cell line. It was observed that the co-treatment of H1299 cells with EGCG and tolcapone or entacapone resulted in a greater than additive, and in some instances synergistic growth inhibitory effect. The mechanism by which this effect was derived cannot be fully elucidated by the current study, though it is expected that tolcapone and entacapone can enhance the chemopreventive activity of EGCG by inhibiting its methylation via COMT. Additionally, both tolcapone and entacapone exhibited significant cancer chemopreventive properties when used singly, and these effects additionally contribute to the activity of the combination.

The results of this study support the hypothesis that COMT status modulates the cancer chemopreventive activity of the tea catechin EGCG. Furthermore, such evidence provides a mechanism-based rationale for the potential development of cancer chemopreventive regimens using EGCG, or EGCG analogs, in conjunction with COMT inhibitors such as tolcapone or entacapone.

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I. Introduction

Tea

Tea, produced from the leaves of *Camellia sinensis,* was initially utilized for its medicinal properties nearly 5,000 years ago.[1] Current worldwide consumption of tea as a beverage is second only to that of water, with approximately three billion kilograms being processed and consumed annually.[2,3] Although there are over 300 varieties, teas can typically be classified as either green, black, or oolong, with the relative global consumption of each being 20%, 78%, and 2%, respectively.[4,5,6]

Green tea is produced by steaming and drying fresh tea leaves at elevated temperatures, preserving the characteristic polyphenols called catechins (Figure 1-1) through the heat-induced inactivation of polyphenol oxidase (PPO).[7] Black tea is produced by crushing tea leaves, facilitating a PPO-mediated oxidation process known as fermentation. During this fermentation process, about 75% of the catechins present undergo enzymatic oxidation and polymerization, forming compounds such as theaflavins and thearubigins.[3] Oolong tea is prepared by inducing a partial fermentation, achieved by crushing just the outer perimeter of tea leaves. Accordingly, oolong tea exhibits characteristics intermediate to both green and black tea, while also containing unique phenolic oligomers such as theasinensin.[8] Over 96 phenolic compounds have been detected in tea samples.[9] Though many of these compounds exhibit bioactivity, this thesis will focus on the properties of the green tea catechins, especially (-)epigallocatechin-3-gallate (EGCG) (Figure 1-1).

The chemical composition of tea varies based on several factors, including the climate it is grown in, the season during which it is harvested, the horticultural production methods utilized to cultivate it, the *Camellia sinensis* variety planted, and the age at which it is harvested.[10] Additionally, the formulation and storage conditions of ready-to-drink tea products can greatly

impact the stability of catechin constituents.[11] The catechins present in tea leaves are also subject to degradation during typical dry-storage conditions, with an average reduction in the catechin content of 32% during a six month storage period.[12]

Despite the above observations, generalizations can be made regarding the chemical composition for each type of tea. For example, a black tea beverage brewed with 1 g of leaf material may contain 30-100 mg of catechins, whereas an equivalent green tea beverage may contain 74-150 mg of catechins.[5] Oolong teas typically exhibit a catechin content intermediate to that of black and green tea.[4]

Catechins

Catechins exhibit a di- or tri-hydroxyl group substitution on the B ring, with a meta-7,7-dihydroxy substituition on the A ring (Figure 1-1).[6] The four major green tea catechins are EGCG, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epicatechin (EC). EGCG is the predominant catechin present in green tea, representing 50-70% of the total catechin content.[8] EGC, ECG, and EC account for the majority of the remaining catechin content, with catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate, 3-*O*-methyl EC/EGC, catechin gallate, and gallocatechin gallate also being present, but to a lesser degree.[6]

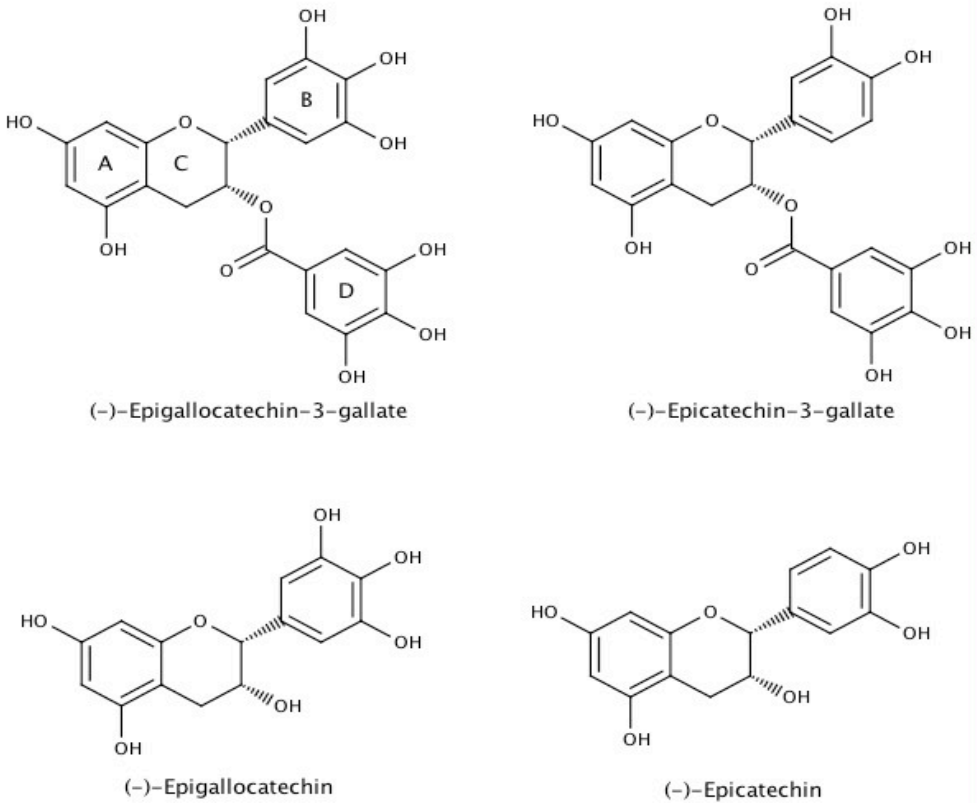

Figure 1-1 Chemical structures of the four major tea catechins

EGCG Biotransformation and Bioavailability

Phase II Metabolism

Phase II metabolizing enzymes are present in most tissues and organs within the human body. Typically, these enzymes serve to detoxify various xeno- and endobiotics. This detoxification process is achieved through several means, depending upon the specific phase II enzyme superfamily. For example, uridine 5'-diphospho-glucuronosyltransferases and sulfotransferases increase the polarity of target compounds through conjugation with glucuronide or sulfate groups, respectively. This increases the hydrophilicity of target compounds, which limits distribution and facilitates excretion via urine and/or bile.[13] Catechol-*O*-methyltransferase (COMT), on the other hand, reduces the activity/toxicity of catechol-containing xenobiotics through the magnesium-dependent methylation of one of the aromatic hydroxyl groups.[14]

Several enzymological and *in vivo* studies indicate that EGCG is readily methylated by COMT, forming 4"-*O*-methyl-(-)-EGCG and 4',4"-*O*-dimethyl-(-)-EGCG. The dimethylated product is the predominate metabolite formed upon exposure to low concentrations of EGCG, whereas the monomethylated metabolite is the major product formed upon exposure to higher concentrations of EGCG.[16,17,18,19] It should be noted that 4'-*O*-methyl-(-)-EGCG is the intermediate with regards to the formation of 4',4"-*O*-dimethyl-(-)-EGCG.[17] In addition to methylation, various UDP-glucuronosyltransferases have been shown to exhibit activity towards EGCG, with EGCG-4"-*O*-glucuronide being the most abundant product.[18] Sulfotransferases also exhibit activity towards EGCG, resulting in various EGCG-sulfate metabolites.[19] Some metabolites are further metabolized by other enzymes to form various mixed EGCG conjugates.[20] The several phase II reactions applicable to EGCG metabolism are depicted in Figure 1-2.

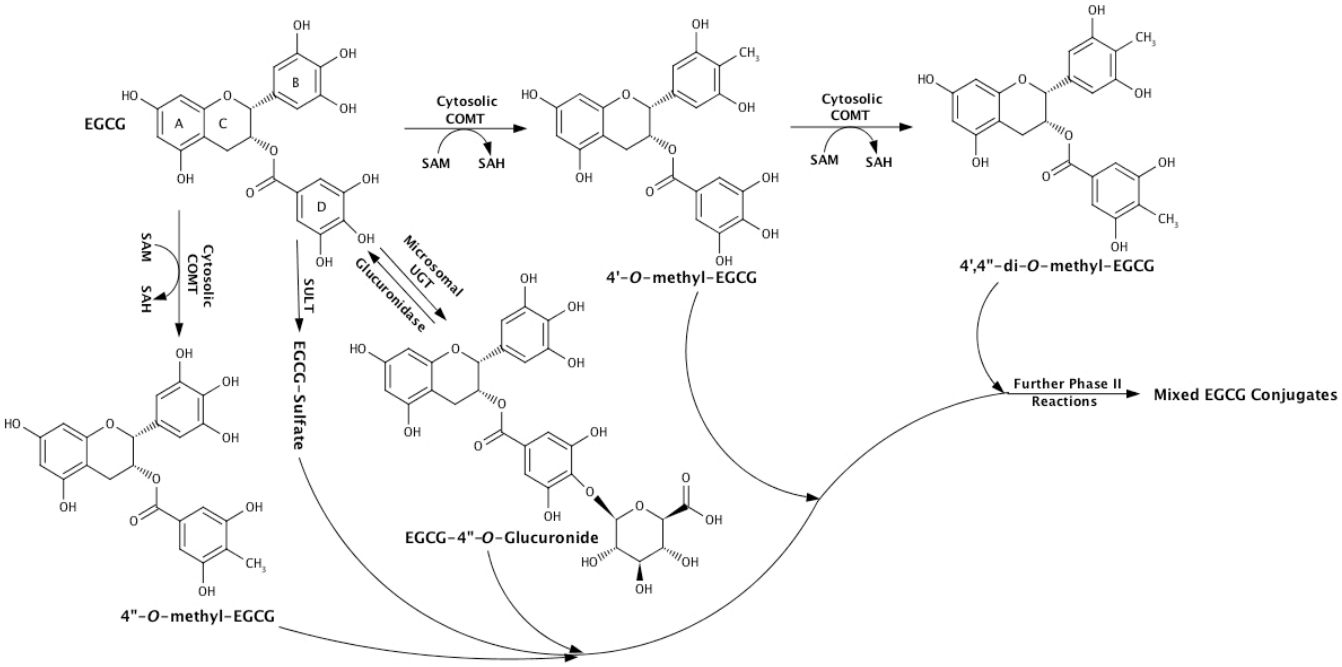

Figure 1-2: Biotransformation of EGCG by phase II metabolizing enzymes. Note: EGCG, (-)-epigallocatechin-3-gallate; COMT, Catechol-*O*-methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

Phase III Transportation

Phase III transporters play an essential role in regulating the distribution and excretion of potentially toxic xeno- and endobiotics.[13] The multidrug resistance related proteins (MRPs) are one such group of transporters. Belonging to the ATP binding cassette superfamily of transport proteins, MRP transporters actively efflux phase II metabolites, and non-conjugated compounds if glutathione is present, from cells, thus limiting cellular exposure to potentially toxic compounds.[21] Many cancerous tissues exhibit elevated MRP expression levels, conferring resistance to anti-cancer agents by limiting the cellular exposure to these compounds.

The distribution of MRP transporters within the human body varies depending on the specific MRP subfamily. MRP1 is ubiquitously expressed throughout the body, though its expression is particularly high in the kidney and lung. It is distributed within the basolateral membrane of cells, where it transports its substrates from the cellular cytoplasm into the interstitial space. MRP2 is localized to the apical membrane of polarized cells within the liver, kidney, and intestine, where it transports its substrates from the cellular cytoplasm into the bile canalicular lumen, the renal tubule lumen, or the intestinal lumen, respectively. [21]

Both MRP1 and MRP2 are thought to play a role in modulating the bioavailability of EGCG, as treatment of MRP1 or MRP2-overexpressing Madin-Darby canine kidney cells with a MRP1 or MRP2 inhibitor, respectively, results in a significant intracellular accumulation of EGCG, 4"-*O*-methyl-EGCG, and 4',4"-di-*O*-methyl-EGCG. [22] Furthermore, treatment of HT-29 human colon cancer cells with an MRP inhibitor results in significant intracellular accumulation of EGCG, EGCG-methylated conjugates, and EGCG-glucuronidated metabolites.[23]

Cellular Uptake of EGCG

In vitro and *in vivo* evidence suggests that cellular absorption of EGCG is accomplished through passive diffusion. The uptake of EGCG by HT-29 human adenocarcinoma cells exhibits

a concentration-dependent linear relationship.[23,24] Furthermore, after administering a single dose of EGCG to CF-1 mice, EGCG exhibits a linear dose relationship within plasma, though EGCG levels plateaued in the small intestine and colon.[24]

It has also been suggested that EGCG uptake may occur by facilitated diffusion via proton-linked monocarboxylate transporters (MCTs), as inhibition of MCT-1 by phloretin or benzoic acid within Caco-2 human colorectal adenocarcinoma epithelial cells has been shown to significantly reduce the cellular uptake of EGCG.[25] However, others have shown that EGCG is a relatively poor substrate for MCTs, as the apical to basolateral transepithelial flux of EGCG through Caco-2 cells is much lower than that of several known substrates, such as ferulic acid.[26] Also, the structure of EGCG is quite different than that of characterized MCT substrates, as MCT substrates are expected to require a monoanionic carboxyl group in conjunction with a nonpolar side chain or aromatic hydrophobic portion. Though the role of MCT-1 in facilitating EGCG transport is not fully resolved, it should be noted that MCT-1 is ubiquitously expressed within the human body.[27,28]

Processes Modulating Bioavailability

Figure 1-3 depicts a proposed model for the discussed parameters which modulate the bioavailability of EGCG, adapted from Lambert et al.[30] After consumption of tea, or green tea extract, a small portion of the ingested EGCG is absorbed by intestinal enterocytes via passive or facilitated (MCT-1) diffusion. A portion of the ingested EGCG is metabolized by microorganisms within the intestinal lumen to form several ring fission products, which are excreted as fecal matter or absorbed by enterocytes via passive diffusion.[4] A portion of the enterocyte intracellular EGCG is subject to phase II metabolism, while some remains in the non-conjugated form. EGCG conjugates (EGCG-X) and non-conjugated EGCG can be actively effluxed from the enterocyte back into the intestinal lumen via MRP2, or into the interstitial

space/blood stream via MRP1. Additionally, the enterocyte intracellular EGCG and EGCG-X can enter the interstitial space/blood stream via passive diffusion.

Once in the blood stream, EGCG and EGCG-X enter systemic circulation via the portal vein, with most of the EGCG being in the non-conjugated form. In hepatocytes and renal cells, EGCG and EGCG-X are subject to the same phase II transformations as in the enterocyte – EGCG can be conjugated, and EGCG-X can be further conjugated. In hepatocytes, EGCG and EGCG-X are actively transported by MRP2 into the bile canaliculus, which ultimately leads to the fecal excretion of these compounds. Most of the EGCG in bile is in the conjugated form. In renal cells, EGCG and EGCG-X are actively transported by MRP2 into the proximal tubule lumen, which leads to the excretion of these compounds via urine. Of the EGCG present in systemic circulation, only a relatively small portion is excreted via urine.

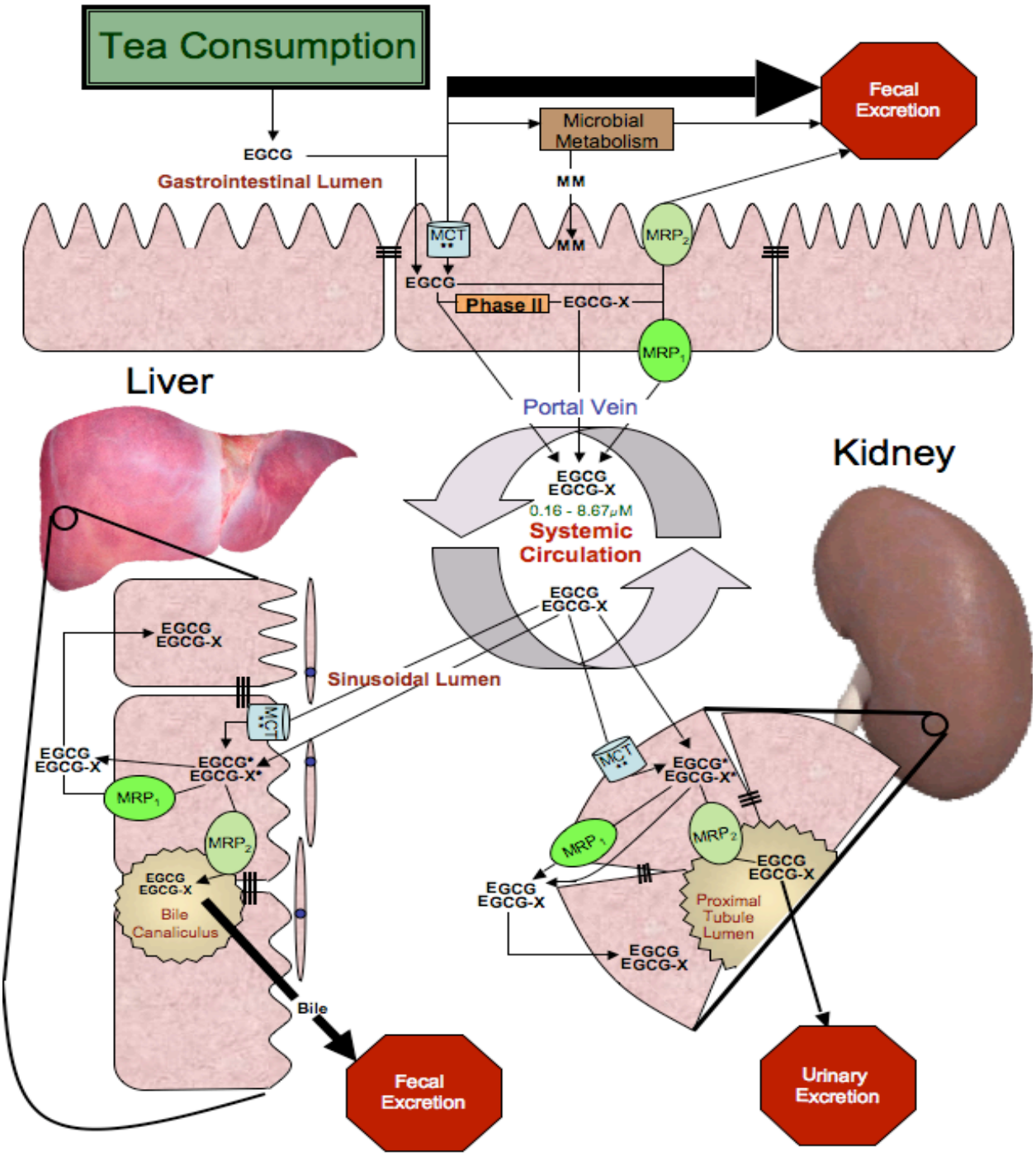

Figure 1-3: Factors modulating the bioavailability of EGCG. The indicated systemic circulation concentration range represents the peak plasma concentration after consumption of about 2 cups of green tea (lower value), or trough plasma concentration after chronic consumption of 1,200 mg of EGCG in a pharmacological dose (higher value). The weight of the arrows indicating routes of excretion represent the degree to which consumed EGCG is excreted via a particular route. Arrows directly entering or exiting a cell indicate passive diffusion. Arrows going through proteins represent protein-mediated transport. **Abbreviations** – EGCG-X: Conjugated EGCG, EGCG: Non-conjugated EGCG, MM: Microbial Metabolites, MCT: Monocarboxylate Transporters, MRP: Multidrug Resistance Proteins.

* Subject to further phase II metabolism

** Role in EGCG facilitated diffusion not entirely elucidated; EGCG is either a substrate or an inhibitor.

Pharmacokinetics

The pharmacokinetic parameters of EGCG have been studied in mice, rats, and humans.[29] It has been suggested that mice are more similar to humans with regards to catechin biotransformative capability and bioavailability than are rats.[30,31,32] After normalizing observed exposure (AUC) values according to the utilized dose, the AUC for humans, mice, and rats has been shown to be 777.8 x 10^3, 713.3 x 10^3, and 80.9 x 10^3, respectively.[32] The absolute bioavailability of EGCG is 0.1% and 26.5% for rats and mice, respectively.[31,32]

A study of the pharmacokinetics of EGCG in humans following a single oral dose of green tea solids (20 mg tea solids/kg) or EGCG (2 mg/kg) found that at T_{max} (1.6hrs), only 0.16% and 0.1% of the ingested EGCG dose, respectively, was present in circulating plasma.[33] One hour after ingestion, 77% of the plasma EGCG was in the free form. This figure was reduced to 64% after 5 hours. EGCG was not detected at measurable levels in the excreted urine.[33]

In another study, consumption of a single 200 mg dose of EGCG in capsulated form by healthy humans, which is the approximate EGCG content contained in 2 cups of brewed green tea, resulted in a peak plasma concentration of 0.16μM.[34] In the same study, consumption of a single 800 mg EGCG dose resulted in a peak plasma concentration of 0.95 μM, demonstrating a dose-response relationship. The time to reach peak plasma concentrations at the 200 and 800 mg dosage levels was 127 and 250 minutes, respectively, with respective half-lives of 118 and 114 minutes.[34] Consumption of a single 800 mg EGCG dose daily for 4 weeks resulted in a 66% increase in EGCG peak plasma concentration, with an increase in the area under the plasma EGCG concentration-time curve from 95.6 to 145.6 μg/mc min.[35] This dose regiment was evaluated as being safe and well-tolerated in healthy human beings.[35]

Peak plasma concentrations of EGCG are influenced by the fasting status of test subjects at the time of EGCG consumption.[36] Under non-fasting conditions, peak EGCG plasma

concentrations of 0.64 and 2.01μM were observed at EGCG dosage levels of 800 mg and 1,200 mg, respectively. After fasting for at least 6 hours, the peak plasma concentrations at equivalent doses were 3.32 and 7.36 μM, respectively.[36] Though the utilized dosage levels were generally well-tolerated, mild and transient nausea was reported by some participants, mostly at the 1,200 mg dosage level during fasting conditions. Accordingly, it was suggested that under fasting conditions, this dosage level may not feasible for chronic EGCG use.[36]

Recently, a phase I trial of polyphenon-E consumption by patients with chronic lymphocytic leukemia examined optimal chronic EGCG dosing.[37] Patients were treated with 400 – 2,000 mg EGCG twice per day under non-fasting conditions for up to six months. The trough plasma concentration, representing the lowest observed concentration, was obtained after one month of treatment and ranged from 2.9 to 3,974 ng/ml (<0.01 to 8.67 μM). Interestingly, trough plasma levels did not exhibit a dose response relationship and did not correlate to the probability of observing a clinical response. Utilizing the NCI Common Terminology Criteria for Adverse Events (version 3.0), which characterizes adverse events on a 1-5 scale with 1 representing a mild adverse event and 5 representing a death-related adverse event, the most severe toxicity grade experienced by more than 80% of the patients was ≤ grade 1, with only 6% of patients experiencing a toxicity grade greater than grade 2 (grade 3, severe adverse event).[37] Accordingly, an EGCG dose contained in polyphenon-E of ≤ 2,000 mg administered twice daily under non-fasting conditions is expected to be generally well tolerated by human subjects.[37]

Catechol-O-Methyltransferase as a Drug Target

As previously discussed, COMT is a phase II metabolizing enzyme that reduces the activity/toxicity of catechol-containing compounds through the magnesium-dependent transfer of methyl groups from S-adenosyl-L-methionine (SAM) in a lysine catalyzed reaction.[14,38]

COMT occurs in two forms within mammals, a soluble (S-COMT) and a membrane-bound form (MB-COMT), both of which are encoded by a single gene.[39,40] S-COMT is the predominate form within most human tissues, though MB-COMT accounts for 70% of the COMT within the brain.[41] The activity of COMT is greatest in the liver, with a relatively high activity in the kidneys and gastrointestinal tract.[42] S-COMT and MB-COMT are located intracellulary and localized primarily to the cytoplasm and rough endoplasmic reticulum, respectively.[43] Within humans, a polymorphism of the COMT encoding gene at codon 108/158 (S-COMT/MB-COMT) results in a Met→ Val substitution, with the Met substituted variant (L) being associated with decreased activity compared to the Val form (H).[44,45] Accordingly, humans can exhibit a $COMT^{H/H}$, COMT $^{H/L}$, or COMT $^{L/L}$ genotype, corresponding to high, intermediate, and low COMT activity phenotypes, respectively.

The nitrocatechols tolcapone and entacapone, depicted in figure 1-4, are both clinically prescribed inhibitors of COMT used in the treatment Parkinson's disease (PD).[46] PD is a chronic neurodegenerative disease associated with the accelerated deterioration of nigral cells, along with concurrent decline and loss of striatial dopamine levels.[46] The decrease in striatial dopamine results in the characteristic motor-function fluctuations associated with PD. To treat PD, the dopamine precursor L-dopa is typically prescribed along with a peripheral inhibitor of aromatic amino acid decarboxylase (AADC), which serves to prevent the peripheral decarboxylation and inactivation of L-dopa.[46] COMT also plays a key role in the metabolism and inactivation of L-dopa. COMT-mediated methylation of L-dopa results in the formation 3-*O*-methyldopa (3-OMD) and reduces the striatial availability of L-dopa. Accordingly, the COMT inhibitors tolcapone and entacapone, which were first characterized in 1989, have been developed for utilization along with L-dopa and AADC inhibitors to treat the motor-function symptoms associated with PD.[46]

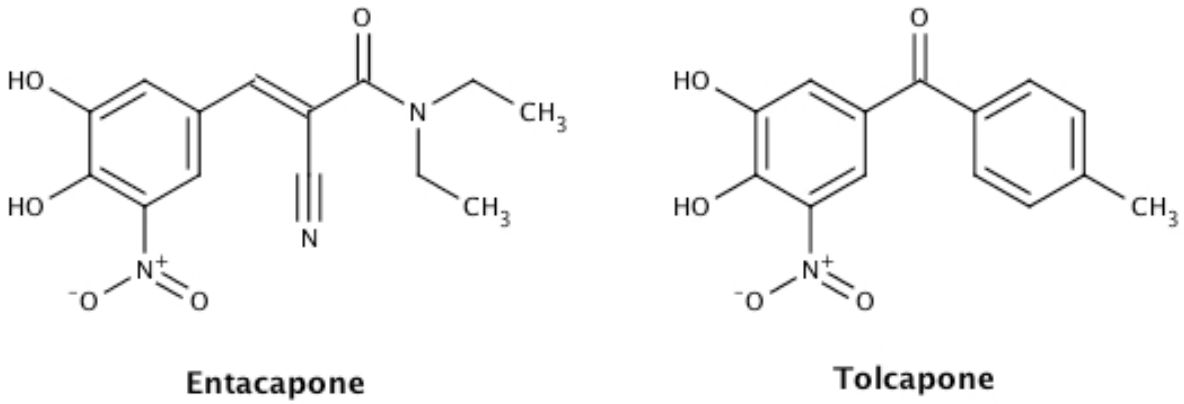

Figure 1-4: The chemical structures of entacapone and tolcapone.

Both tolcapone and entacapone are competitive inhibitors of COMT, but whereas tolcapone is capable of crossing the blood-brain barrier and can inhibit both peripheral and central nervous system-located COMT, entacapone is primarily a peripheral-acting COMT inhibitor.[46] Tolcapone also exerts a greater COMT inhibitory effect than entacapone, as entacapone and tolcapone exhibit IC_{50} values of 773 and 155 nM, respectively, for the inhibition of human liver COMT-mediated methylation of 3,4-dihydroxybenzoic acid, which has a K_m value of 19.9 μM.[48,48] Additionally, tolcapone exhibits a greater bioavailability than entacapone. A 200 mg (oral) dose of tolcapone or entacapone in humans results in a C_{max} of 23.1 and 5.9 μM, respectively, and an AUC of 18.5 and 1.6 μg.h/ml, respectively.[46]

Tolcapone and entacapone also have unique toxicological profiles. Whereas entacapone is generally well-tolerated at pharmacological doses, tolcapone exhibits a higher degree of hepatoxicity, possibly through the uncoupling of oxidative phophorylation within mitochondria.[46] Furthermore, the use of tolcapone has been associated with several incidences of fatal fulminant hepatitis.[46] Though still clinically prescribed, the use of tolcapone requires extensive monitoring for several hepatoxicity-associated biomarkers within those to which it is administered.

Tea Catechins and Cancer

Chemopreventive Properties

Tea catechins have been studied extensively for their cancer chemopreventive properties both *in vitro* and *in vivo*.[2-4,6-8, 50-53] In this regard, EGCG has been implicated as the most bioactive of the tea catechins. Potential mechanisms for the anticancer properties of EGCG include inhibition of carcinogen activation, induction of certain phase II enzymes (*e.g.* quinone reductase), inhibition of cell growth, induction of cell cycle arrest, induction of apoptosis, inhibition of inflammation, inhibition of angiogenesis, and inhibition of metastasis. EGCG

inhibits the following cellular targets and signaling pathways relevant to the above discussed modes of action: the nuclear factor-κB pathway, the mitogen activated protein kinases and activator protein-1 pathway, the epidermal growth factor receptor-mediated pathway, the insulin-like growth factor-I-mediated pathway, proteasome activity, vascular endothelial growth factor activity, matrix metalloproteinase activity, and urokinase-plasminogen activator activity.

Though EGCG has been shown to modulate these targets *in vitro*, many have not been demonstrated to be prevalent *in vivo*. Furthermore, the EGCG concentrations shown to be efficacious *in vitro* are often much greater than those actually experienced *in vivo*. Despite this, EGCG has been shown to exert chemopreventive properties *in vivo* in the lung, oral cavity, esophagus, stomach, small intestine, colon, skin, prostate, breast, liver, bladder, pancreas, and thyroid. [2-4,6-8]

Modulation by COMT

Several studies have suggested that COMT plays a role in modulating the cancer chemopreventive properties of EGCG, as methylated-EGCG conjugates have been shown to exhibit depressed anticancer activity relative to EGCG. Compared to EGCG, 4"-*O*-methyl-(-)-EGCG and 4'-*O*-methyl-(-)-EGCG both demonstrate decreased growth inhibitory and pro-apoptotic activity within murine osteoclasts, possibly due to the decreased Fe(III)-reducing ability of the methylated-EGCG conjugates.[54] Furthermore, the capacity 4"-*O*-methyl-(-)-EGCG to inhibit cellular proliferation, block NF-κB activation, and induce apoptosis was significantly lower than that of EGCG within the human prostate cancer cell line LNCaP.[55] Also, the ability of EGCG to inhibit the activity of purified rabbit proteasome decreases as its degree of methylation increases.[56] Similarly, the capacity of EGCG to inhibit proteasome activity within breast cancer MDA-MBA-231 cells expressing a high activity COMT phenotype (COMT $^{H/H}$) is augmented when EGCG is used in conjunction with the COMT inhibitor 3,5-dinitrocatechol.[57,58]

Recently, the interrelationship between tea intake, COMT genotype, and breast cancer risk was examined within a population-based case-control study of breast cancer in Chinese-, Japanese-, and Filipino-American women.[59] The study indicated that tea-drinking individuals and non-tea-drinking individuals homozygous for the high activity COMT allele (COMT $^{H/H}$) did not differ with regards to breast cancer risk. However, an inverse association between tea intake and breast cancer risk was demonstrated for individuals possessing at least one low activity COMT allele (COMT $^{H/L}$ or COMT $^{L/L}$), suggesting that the *in vivo* methylation of tea catechins via COMT diminishes the cancer chemopreventive properties of the catechins.[59]

Purpose

The purpose of the present study is to further examine the role of COMT in modulating the chemopreventive properties of EGCG, accomplished by determining the growth inhibitory spectrum of EGCG with either tolcapone or entacapone within H1299 human lung cancer cells. It is hypothesized that co-treatment with tolcapone or entacapone will enhance the anticancer capacity of EGCG in this cell line.

II. Materials and Methods

Materials

EGCG (93% pure) was purchased from Taiyo GreenPower Co. (Jiangsu, China). Entacapone and tolecapone (99% pure) were purchased from Synfine Chemical Co. (Ontario, Canada). For experimental purposes, a 100 mM stock solution of each compound was prepared in dimethylsulfoxide and stored at -80^{o}C. Superoxide disumutase, catalase and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade commercially-available.

Cell Culture

Human lung adenocarcinoma cell line H1299 was obtained from American Type Tissue Culture Collection (Manassas, VA). Cells were maintained in log-phase growth in RPMI 1640 medium (MediaTech, Manassas, VA, USA) supplemented with 10% heat inactivated fetal bovine serum and 100 U/ml of penicillin at 37°C in 95% humidity and 5% CO_2. Cells were sub-cultured twice per week.

Cell Treatments

Cells were seeded in 96-well plates at 5 x 10^3 cells per well, and were incubated for 24 hours at 37°C. Cells well were then treated with serial concentrations (0-50 μM) of EGCG, EGCG + tolcapone (1:1), EGCG + entacapone (1:1), tolcapone, or entacapone in 100μl of complete medium containing 60 U/ml of both catalase and SOD. Catalase and SOD were included to prevent auto-oxidation of EGCG under cell culture conditions. Cells were incubated for 24 hours at 37°C in 95% humidity and 5% CO_2. After the 24-hour treatment incubation period, the old medium was removed and cells were washed once with complete medium. Cells were then incubated with 100μl of complete medium containing 1 mg/ml of MTT and incubated

for two hours. The MTT-containing medium was removed, and the resultant formazan precipitate was solubilized in 100μl of DMSO and the absorbance was measured at 540 nm using a MicroPlate Auto-reader (Bio-tek Instruments).

Data Analysis

Absorbance values for treated cells were normalized to the average absorbance of vehicle-treated cells. All results were confirmed in 3-4 independent experiments. Dose-response curves were prepared using Microsoft Excel. The median inhibitory concentration (IC_{50}) values were determined by regression analysis. The median-drug effect analysis, described by Chou and Talalay, was implemented with modifications to qualify potential synergy between EGCG and tolcapone or entacapone based on a combination index (CI).[60] This model was used to calculate the slope coefficient (m) of the dose response curves by plotting the response, $\log(F_a/F_u)$, and regressor, log(d) (not depicted), where F_a and F_u correspond to the fraction of cells affected (% growth inhibition) and unaffected, respectively, at a certain dose (d).

The equation $D_x = D_m(F_a/F_u)^{1/m}$ was used to calculate the doses, $(D_x)_1$, $(D_x)_2$, and $(D_x)_{1,2}$ corresponding to a given F_a level for compounds 1, 2, and their combination, respectively. For mutually exclusive compound interactions, $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, whereas $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + [(D)_1(D)_2]/[(D_x)_1(D_x)_2]$ for mutually nonexclusive compound interactions, where $(D)_1$ and $(D)_2$ correspond to the proportional composition of $(D_x)_{1,2}$ with regards to compounds 1 and 2, respectively. CI values were calculated based on both mutually exclusive and mutually non-exclusive interaction parameters, as the relationship between cellular targets of EGCG relative to that of tolcapone and entacapone is not fully resolved. The CI value was utilized to qualify potential synergy on the basis that a $CI < 1$ indicates synergy, a $CI = 1$ indicates additivity, and a $CI > 1$ indicates antagonism.[60] CI values were calculated for F_a levels ranging from 50-99% inhibition, as effect levels lower than 50% are not generally clinically relevant.

III. Results

Growth Inhibition

Figure 3-1 depicts the response of H1299 cells to treatment with EGCG or tolcapone alone or in combination. Treatment with EGCG had little to no effect on cell viability at all treatment levels except for at the 50 μM level, at which EGCG exhibited a growth inhibitory effect of approximately 13%. By contrast, treatment with the combination of EGCG and tolcapone reduced cellular viability in a dose-dependent fashion with an IC_{50} of 50.2 μM (25.1 μM EGCG+25.1 μM tolcapone). The tolcapone treatment exhibited a growth inhibitory effect intermediate to that of EGCG and EGCG+tolcapone, with an IC_{50} of 33.8 μM.

Similarly, treatment with the combination of EGCG and entacapone also reduced the number of viable cells in a dose-dependent manner (Figure 3-2). The EGCG + entacapone treatment exhibited greater growth inhibitory effects than treatment with either compound alone (IC_{50} = 57.6 μM; 28.8 μM EGCG+28.8 μM entacapone). The entacapone treatment also exhibited a growth inhibitory effect (IC_{50} = 38.9μM). The IC_{50} of EGCG, extrapolated via the regression depicted in Figures 3-3 and 3-4, was 227.3 μM.

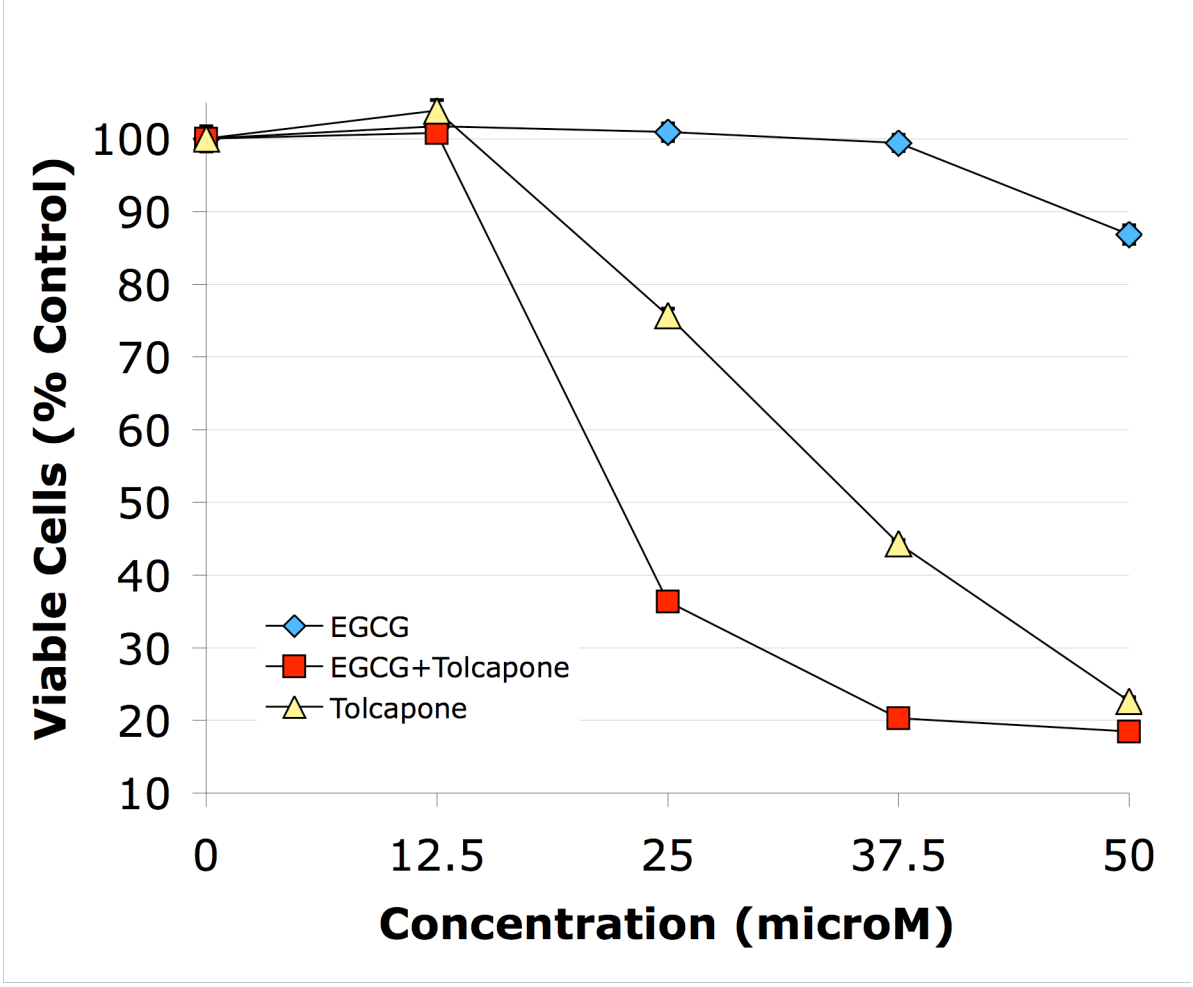

Figure 3-1: Dose response figure depicting the viability of H1299 cells treated with EGCG, EGCG+Tolcapone (each present at the indicated concentration), or Tolcapone. Error bars represent the standard error at each data point.

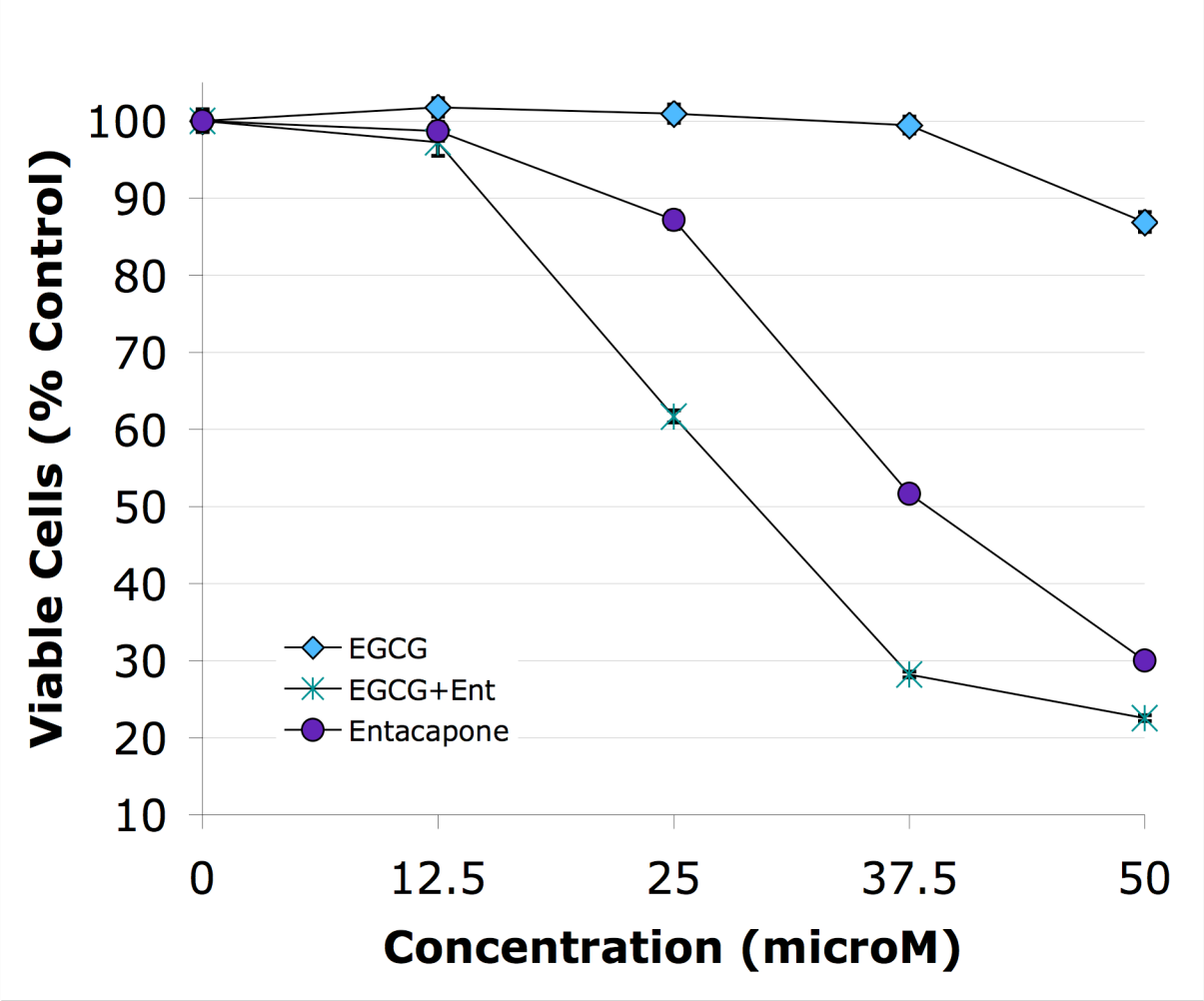

Figure 3-2: Dose response figure depicting the viability of H1299 cells treated with EGCG, EGCG+Entacapone (each present at the indicated concentration), or Entacapone. Error bars represent the standard error at each data point.

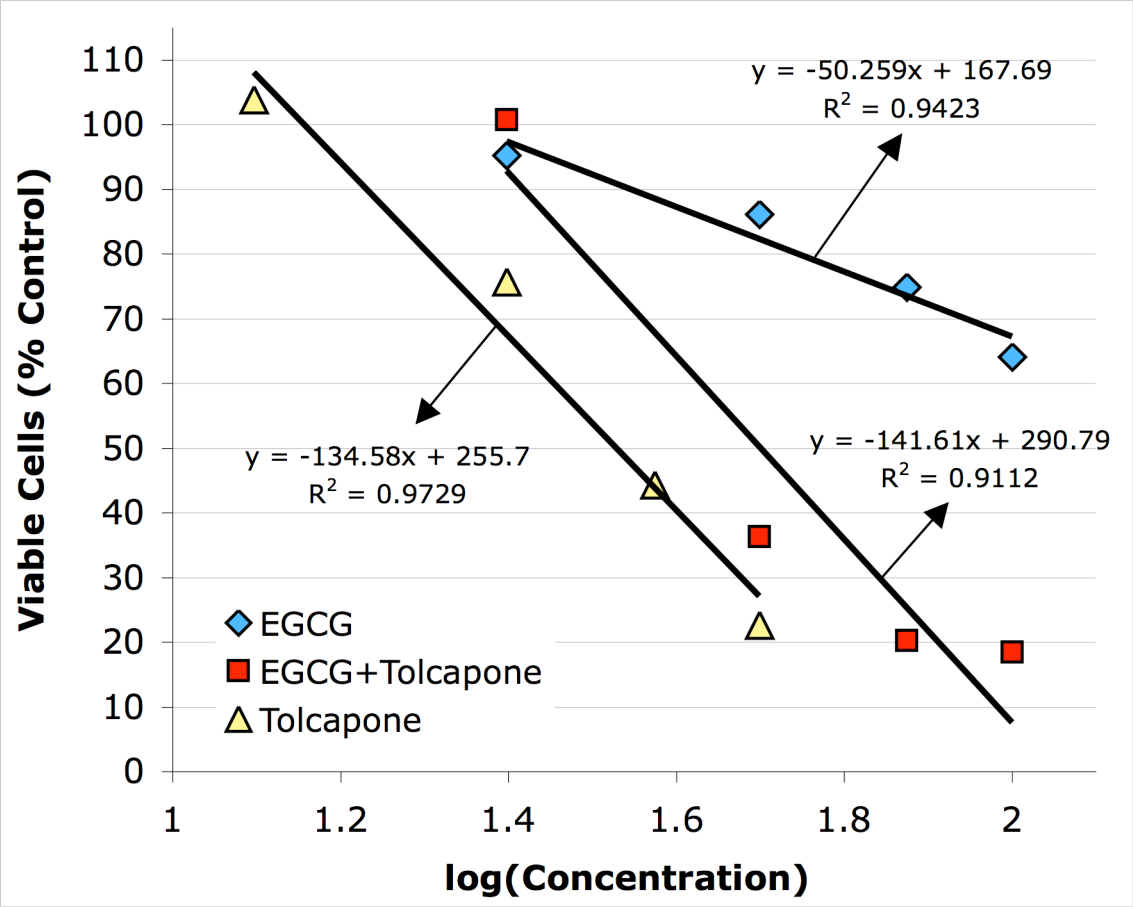

Figure 3-3: % Viability as a function of the log(concentration) for EGCG, EGCG+Tolcapone, and Tolcapone data, along with resultant regression equations and coefficients of determination.

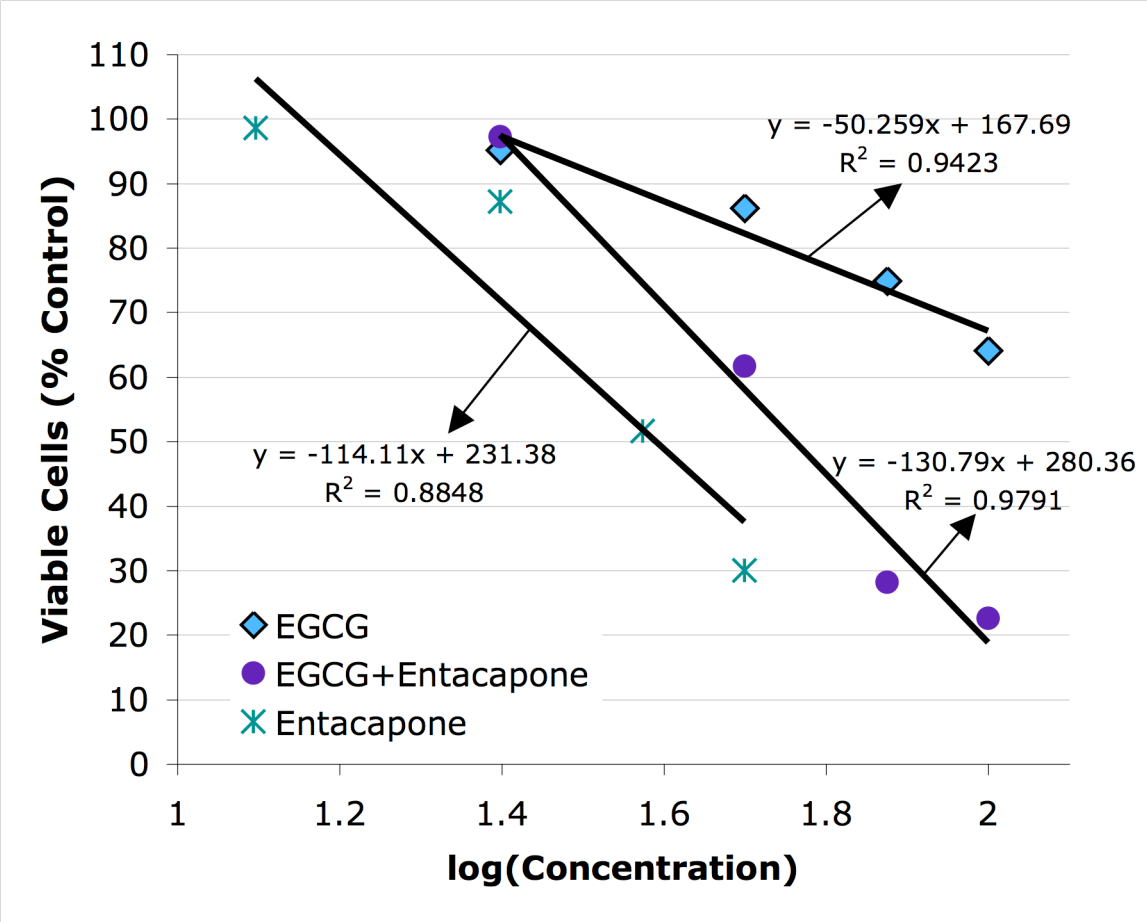

Figure 3-4: % Viability as a function of the log(concentration) for EGCG, EGCG+Entacapone, and Entacapone data, along with resultant regression equations and coefficients of determination.

EGCG and COMT Inhibitor Interaction

The treatment of H1299 cells with the combination of either EGCG and tolcapone or EGCG and entacapone resulted in a synergistic growth inhibitory interaction at growth inhibition levels of 50-99%, as the calculated CI values for this effect range were all less than 1 (Figure 3-5).[60] These effects were observed regardless of whether the values were calculated based on a mutually exclusive or mutually non-exclusive model. CI values calculated based on a mutually exclusive or mutually non-exclusive model for each examined compound combination did not vary by greater than 0.1 at a given F_a level. Furthermore, CI values decreased as a function of F_a, indicating that the degree of synergy between EGCG and tolcapone or EGCG and entacapone increased with increasing levels of growth inhibition.

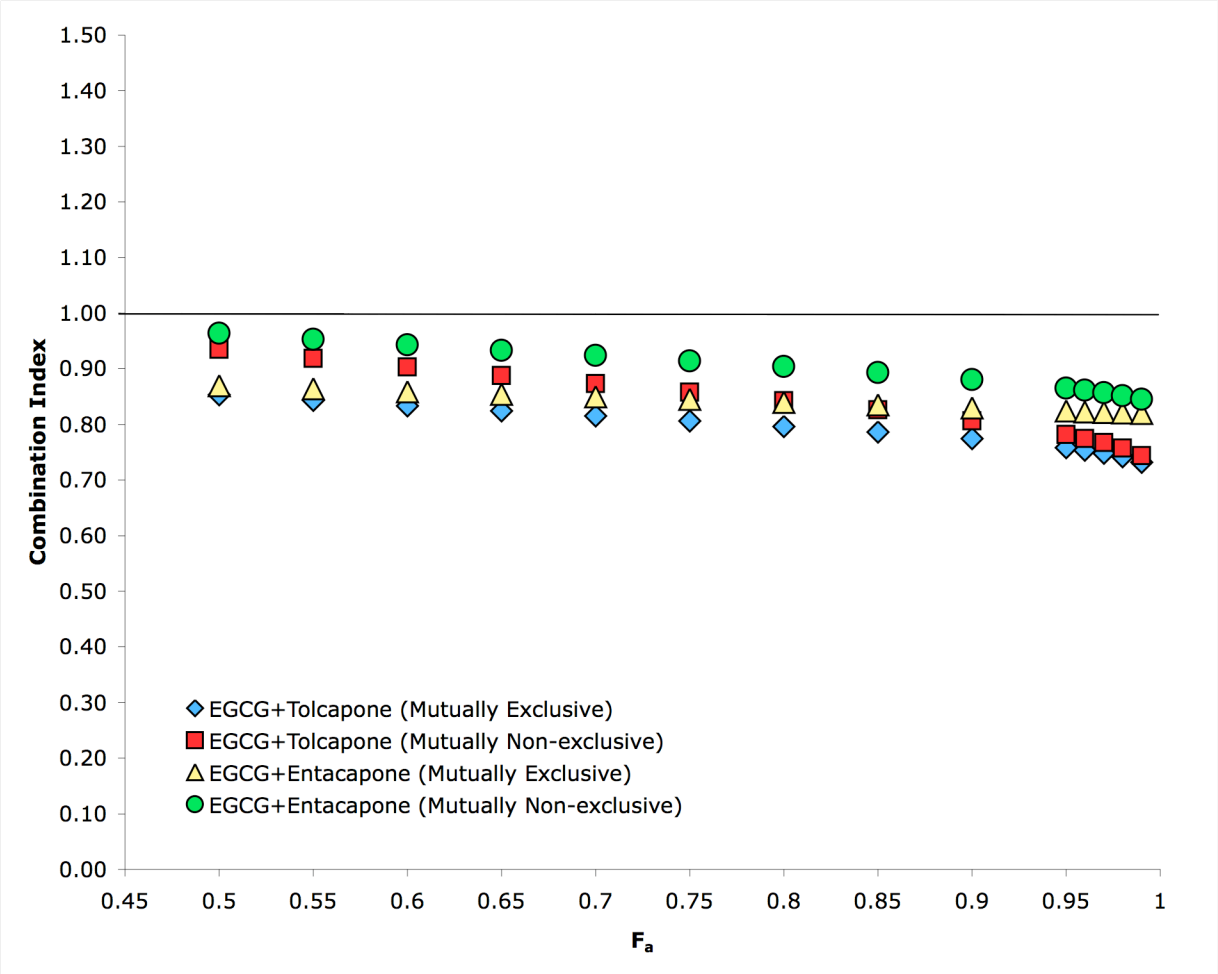

Figure 3-5: Combination Index (CI) Plot of calculated CI values as a function of the fraction of cells affected (F_a). for each examined drug combination, CI values were calculated based upon both a mutually exclusive and mutually non-exclusive parameter.

IV. Discussion

Based on the calculated CI values (Figure 3-5), it can be observed that compared to the compounds used singly, the co-treatment of H1299 cells with EGCG and either tolcapone or entacapone resulted in synergistic growth inhibitory effect at concentrations causing 50-99% growth inhibition. EGCG and tolcapone exhibited a greater inhibitory effect than EGCG and entacapone.

This observation seems to support the hypothesis that the co-treatment of H1299 cells with EGCG and tolcapone or entacapone results in enhanced EGCG chemopreventive properties, and indicates the need for further *in vitro* and *in vivo* experiments. Presently, it cannot be fully ascertained if the observed growth inhibition is the result of the enhanced tolcapone/entacapone-mediated inhibition of COMT or some other mechanism. Previous studies using dinitrocatechol as the COMT inhibitor found no growth inhibitory activity of that compound as a single agent. By contrast, both tolcapone and entacapone showed strong growth inhibitory effects as single agents.[61] It is possible that the synergy reported here is the result of mechanisms independent of effects on COMT. Though it is expected that tolcapone and entacapone are more potent inhibitors of COMT than is EGCG, the discussed discrepancy could be clarified through further studies that monitor the metabolic profile of EGCG in H1299 cells undergoing the various treatment regimens.[48,65] It should be noted that although derivatives of entacapone have been shown to exhibit cancer chemopreventive activity, to the author's knowledge, this is the first time that tolcapone and entacapone have been demonstrated to exert a cancer chemopreventive effect.[61]

As is consistent with many of the *in vitro* studies pertaining to the cancer chemopreventive properties of EGCG, the concentrations shown to be efficacious with regards to inhibiting the growth of H1299 cells are greater than those likely to be observed *in vivo* following the oral ingestion of tea. The highest plasma C_{max} for EGCG observed in the literature

was approximately 8.5 μM, which was achieved through chronic consumption of pharmacological doses of EGCG.[37] The plasma C_{max} for tolcapone and entacapone at dose levels relevant to PD treatment are approximately 23 and 6 μM, respectively.[46] However, the calculated IC_{50} value within H1299 cells for EGCG used in conjunction with tolcapone at a 1:1 ratio was 50.16 μM (25.08 μM EGCG and 25.08 μM tolcapone), and that of EGCG used in conjunction with entacapone at a 1:1 ratio was 57.72 μM (28.86 μM EGCG and 28.86 μM entacapone).

The models used to classify the interaction between EGCG and tolcapone or EGCG and entacapone are expected to have been accurate representations. The lowest r^2 based on the regression utilized to calculate IC_{50} values was 0.88, corresponding to the entacapone regression. The lowest r^2 based on the regression utilized to calculate m values was 0.85, followed by 0.95, corresponding to the EGCG+tolcapone and tolcapone treatments, respectively.

Though there is some question about the underlying mechanisms of synergy, the results of this study largely support other studies implicating COMT as a key modulator of the cancer chemopreventive properties of EGCG.[54-59] Accordingly, it would be interesting to re-evaluate the efficacy of EGCG in other model systems in manner that accounts for COMT status, as it is possible that lack of efficacy corresponds to high levels of COMT in the cell line, animal model, or human subject under study. For example, a recent phase I trial of Polyphenon-E, a green tea extract, in patients with chronic lymphocytic leukemia found no dose-response relationship for plasma levels of tea catechins. Likewise, there was no dose-response relationship for observed clinical response (reductions in absolute lymphocyte count and adenopathic nodal area).[37] Perhaps this lack of an apparent dose-response is related to the COMT activity status of those individuals examined.

V. Conclusion

The co-treatment of human lung cancer H1299 cells with EGCG and the clinically used COMT inhibitors tolcapone or entacapone resulted in a greater than additive, and in some instances synergistic growth inhibitory effect against H1299 cells. Though the exact mechanism by which this effect was derived cannot be fully elucidated in the current study, it is expected that tolcapone and entacapone enhanced the chemopreventive activity of EGCG, in part by inhibiting the COMT-mediated methylation of EGCG. In addition to further experiments to assess the effect of the combination on cell cycle progression and apoptosis, further studies should be devoted to examining the metabolic profile of EGCG in H1299 cells.

The presented results, with previously reported studies, support the hypothesis that COMT status modulates the cancer chemopreventive activity of the tea catechin EGCG. Though requiring further examination for *in vivo* relevance, such evidence provides the mechanism-based rationale for the development of cancer chemopreventive regimens using EGCG, or EGCG analogs, in conjunction with COMT inhibitors such as tolcapone or entacapone.

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Academic Vita

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Education: The Pennsylvania State University - Schreyer Honors College, University Park, PA

Major: B.S. Agricultural Science Minor: Agronomy

Honors: Food Science Credits Completed: 156

Graduation Date: December 18, 2010

Employment:

Pennsylvania Certified Organic — December 2010 - Current

- Implement organic certification policies and procedures consistent with those outlined by the USDA in order to ensure the integrity of organic products in the marketplace
- Facilitate the organic certification process for farmers and food processors

PSU Northeast Sustainable Agriculture Research and Education Team — August - December 2010

- Research Assistant
- Assist research team in all aspects pertaining to a sustainable dairy cropping-system project
- Duties include, but are not limited to, organizing supplies for sampling events, soil sampling and processing, harvesting and processing crop samples, collecting gas emission samples, and conducting population counts

Community Help Centre — August 2010 - December 2010

- Weekend Hotline Staff-person
- Responsible for providing crisis intervention, short-term counseling, and information and referral services for the Community Help Centre lines and numerous off-hours support lines to maintain 24/7 hotline service

Pennsylvania Department of Agriculture, Bureau of Plant Industry — May 2010 - August 2010

- Forest Insect Pest Aid
- Responsible for conducting field surveys for several invasive insect pests and native bees
- Provided extension services to public regarding invasive insect pests

PSU Tomato Molecular Genetics and Breeding Laboratory — May 2009 - October 2009

- Assist with planting, field maintenance, and scouting for pests and diseases
- Utilize molecular techniques to prepare samples and analyze germplasm

Plowshare Produce Community Supported Agriculture — July 2009 - October 2009

- Fieldwork assistant
- Assist in the harvesting of horticultural crops

PSU Adventure Recreation Program — August 2008 - May 2010

- American Red Cross CPR and Wilderness First Aid Certified
- Responsible for coordinating and leading groups of 4-8 students on weekend backpacking, canoeing, and fly-fishing trips throughout Pennsylvania
- Member of Program and Strategic Planning Committee (2008)

ConAgra Foods — May 2008 - August 2008

- Intern within Research, Quality, and Innovation Department
- Conducted research within the meat snack division
- Developed processing techniques to maintain product quality, while drastically reducing production times

Seitz Gourmet Cakes and Catering — 2000 - 2007

- Food Prep/ Baker/ Server
- Honed communication skills through interactions with customers and coworkers

Notable Activities/ Awards:

Gamma Sigma Delta, Member — April 2010 - Current

- Agricultural Honor Society

Undergraduate Soils Teaching Assistant — January 2010 - May 2010
- Co-taught one section of an introductory soils lab
- Collaborated with Soils 101 Teaching Team to review course content, instructional methods, and grading criteria

Community Help Centre Volunteer and Staff Member — May 2009 - Current
- Received extensive crisis and basic needs counseling training (180 hrs)
- Completed a 340 hour volunteer commitment
- January 2010 volunteer of the month for "incredible dependability, professional conduct, and excellent crisis intervention and basic needs skills."
- Member of Pennsylvania 2-1-1 Program Integration Committee
- Member of Executive Director Hiring Committee

PSU Organic Community Garden — April 2009 - Current
- Member

Pennsylvania Association for Sustainable Agriculture — 2009 - Current
- Member

PSU Food and Disease Prevention Research Laboratory — September 2008 – December 2010
- Undergraduate Research Assistant
- Conduct independent research examining the synergistic anticancer properties of the phytochemical EGCG when used in conjunction with commonly prescribed Parkinson's Disease medications

Scholarships and Research Grants — 2008 - 2009
- PSU College of Agriculture Scholarship ($1,700)
- Schreyer Honors College Summer Research Grant ($700)
- Speizer Research Grant ($500)

Rotary Sponsored Exchange Student – Finland — September 2004 - August 2005
- Honorary Rotarian
- Developed strong problem solving and interpersonal skills

Selected Relevant Coursework and Grades:

For samples of my academic work, please review my website at: http://sites.google.com/site/aes5119/home

Global Food Strategies (A)
Agroforestry (A)
Introduction to Soils (A)
Supervised Soils Teaching Experience (A)
Classical Ecology (A)
Honors Plant Pathology (A)
Plant Propagation (A)
Plant Physiology (A)
Molecular and Cellular Biology (A-)
Plant Tissue Culture and Biotechnology (A-)
Forest Herbaceous Plant ID and Ecology (A)
Introductory Food Science (A)
Food Chemistry (A)
Applied Food Microbiology (A)
Physiology of Nutrition (A)
Introduction to Microbiology (A-)
Fundamentals of Organic Chemistry (A)
Experimental Chemistry I (A-)

Plant Ecology (A)
Field Crop Management (A-)
Honors Introduction to Entomology (A)
Field Crop Entomology (A)
Honors Principles of Weed Management (A)
Principles of Agronomic Field Operations (A)
Introduction to International Agriculture (A-)
Biometry (A)
Biology: Populations and Communities (A)
Biology: Basic Concepts and Biodiversity (A)
Rhetoric and Composition (A)
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Food Chemistry Lab (B+)
Applied Food Microbiology Lab (A)
Principles of Immunology (B)
Introduction to Microbiology Lab (A)
Fundamentals of Organic Chemistry Lab (A)
Experimental Chemistry II (A)