

Analysis of the Anticancer Potential of Green Tea's EGCG Component to Inhibit Immortal Cells

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Green tea has a high abundance of catechins and other antioxidants found organically within its composition. In recent studies, a polyphenolic catechin, referred to as EGCG, has shown the potential to promote advancements in the development of non-harmful cancer treatments. Several experimental questions were explored regarding EGCG, including the concentration of EGCG present in commercial green teas, its anticancer potency, and EGCG's potential contributions to cell cycle arrest. Briefly, a green tea extract was prepared utilizing ground green tea leaves and a polar extraction method. The fluorescent properties of EGCG were exploited to quantitate its concentration in green tea preparations in comparison to a purified EGCG standard. The anticancer potency of EGCG was then assessed by exposing HeLa (cancer cells) and MRC-5 (primary) cells to green tea extract- containing the EGCG component- for 24 hrs. The cells were evaluated for viability, suggesting that higher concentrations of extract exerted specificity toward cancer cells over the control model. Lastly, HCT-116 cancer cells were evaluated for cell cycle arrest following drug treatment. Results indicated that green tea extract imposed a cytotoxic effect selective to cancer cells while avoiding cytotoxicity to a primary cell model. In addition, treated cancer cells experienced an increase in the cell population at the G1 phase, indicating a halt in the cell cycle. This hypothesis was supported by an observed decrease in cell population in the S phase. These results show the potential for green tea extracts containing EGCG to be potential components in cancer therapeutics and/or preventatives.

Green tea has been used as a therapeutic in Traditional Chinese medicine since the Han dynasty in 206 BC.¹ Green tea has a high abundance of polyphenols, which are organic compounds that occur naturally in plant-based foods. Polyphenols are regarded as reducing agents with antioxidant properties, along with other naturally occurring compounds in green tea such as vitamin C and vitamin E.² Antioxidants play a critical role in human health by protecting the body's tissues from oxidative stress and chronic diseases, such as cancer.³ In addition, polyphenols have anti-inflammatory and anti-microbial properties, which provide several health benefits for those that include green tea or other polyphenol-containing plants in their diet. The advantages of these promising compounds are a source of ongoing preclinical and clinical research studies.⁴

Polyphenols serve as antioxidants by affecting enzyme functioning⁵ and are capable of neutralizing reactive oxygen species

(ROS) in the body. ROS are formed from partially reduced oxygen molecules that lead to a cellular imbalance inducing oxidative stress.⁶ Cells that experience oxidative stress are prone to cellular damage to lipids, proteins, and DNA⁷ – effects that jeopardize cell viability. Epigallocatechin gallate (EGCG) is an abundant polyphenolic compound naturally found in tea that possesses potent antioxidant properties.⁸ EGCG is found in multiple variations of the *Camellia sinensis* plant, however, but has the highest documented concentration in green tea. As polyphenols possess antioxidant properties that can neutralize these carcinogens, green tea serves as a vehicle for an EGCG component capable of protecting cell integrity and inhibiting uncontrolled cell proliferation in cancer.⁹

In addition to its antioxidant properties, EGCG also may interact directly with cancerous cells in vivo.¹⁰ EGCG demonstrates a high binding affinity to cell-surface proteins commonly found on cancerous cells.¹¹ For example, EGCG can bind to the 67 kDa laminin receptor,¹² which is a non-integrin cell surface receptor that supports cancer cells' metastasis and cancer progression.¹³ By mediating several cellular processes

such as the detoxification of compounds and suppression of MAPK – a signaling complex whose cascade is routinely involved in oncogenesis and tumor resistance¹⁴ - EGCG has the capacity to improve patient outcomes in cancer therapies.

While we know that EGCG may be beneficial, we do not know to what capacity it affects cells on the cellular level.¹⁵ Taking this into consideration, the anticancer potency of EGCG and green tea extract was evaluated in vitro. Several experimental questions were explored regarding EGCG including the concentration of EGCG in commercial green teas,¹⁶ its cellular permeability,¹⁷ and its anticancer potency in cell culture.

Overall, this research project seeks to identify compounds in nature that can reduce cancerous cell populations in the human body. This research may serve to discover a therapeutic mechanism that could be incorporated into supportive or targeted therapies to improve patient outcomes. The goal of this is to minimize the damage to healthy tissues during treatment as compared with less specific oncologic treatments such as chemotherapy¹⁸ and radiation.

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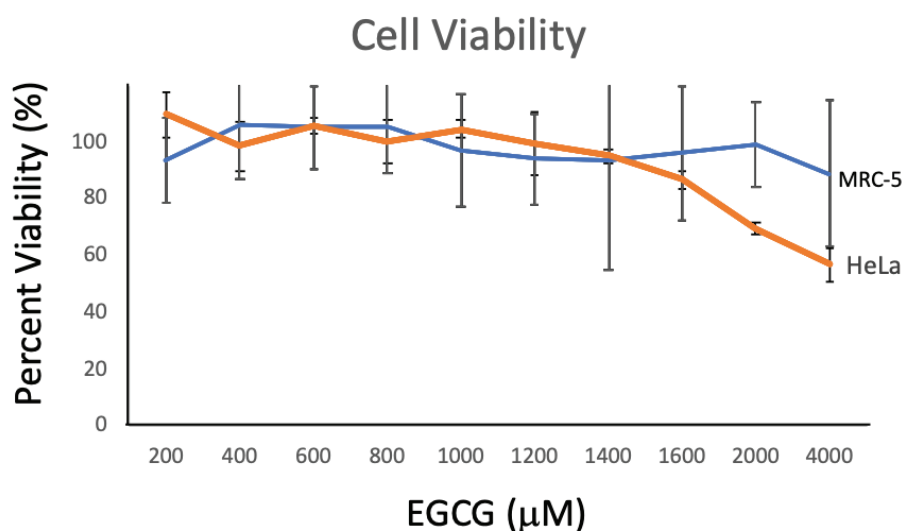


Figure 1 | MRC-5 and HeLa viability in increasing doses of Green Tea Extract containing known concentrations of EGCG. Cells were grown for 24 hours in the presence of the indicated concentration of Green Tea Extract treatment and viability was assessed with MTT after a 24-hour exposure period.

Materials & Methods

Generation of green tea extract

Organic green tea leaves (Vahdam Teas) were manipulated into a medium-ground powder. Since EGCG is a polar molecule, a polar extraction utilizing DI water was conducted to obtain the maximal extraction of EGCG from the leaves. In addition to being an efficient extraction solvent, the usage of water allows for potential correlations to be drawn to human consumption.¹⁹ The ground leaves were allowed to steep for 24 hours before sterile filtering. After filtering, the stock was shielded in a dark cabinet to prevent potential degradation from light exposure.

Quantitation of EGCG in green tea

High-pressure liquid chromatography can separate complex mixtures according to their polarity in RP-HPLC. The evaluation method utilized a C18 column (Thermo Scientific Cat. No. 28105-154630) and was run at 0% B consisting of Acetonitrile (Fisher Scientific Cat. No. AA42311K7) and 0.1% Formic Acid (Cat No. 64-18-6) for 15 minutes utilizing an isocratic method.²⁰ An absorbance sweep was run on an EGCG standard (Millipore Sigma Cat. No. 989-51-5) to reveal an optimal wavelength of 266 nm. Following, a standard curve was run with an EGCG standard to compare EGCG content with the green tea extract. The gradient method for this pro-

cedure incorporated a 0-10 min run at 0% B, followed by a flushing of the column using 100% B. Given limitations on time and budget, an extrapolation of data was made, allowing the extract to be quantitated as 718 mM EGCG.

Cell viability assay with green tea extract

Cell viability was evaluated with the MTT assay.²¹ HeLa cells were plated at 5,000 cells/well in a 96 well plate (Fisherbrand Cat. No. FB012931), and the MRC-5 cell line was plated with 5,000 cells/well accordingly. After seeding, each cell line was allowed 24 hours to firmly attach to the plate. At the 24-hour mark, green tea extract was introduced to the cells and incubated for another 24 hours. After drug exposure, the extract was removed and a working dilution of 5 mg/mL of MTT solvent (Biosynth Cat. No. 298-93-1) dissolved in PBS was added to the cells for a 4-hour incubation. DMSO (Fisher Chemical Cat. No. 67-68-5) was used to lyse the cells and expose the converted crystals while incubating for 15 minutes with light shaking. The plate was then read at 570 nm on a SpectraMax M3 to examine cell viability.

Flow cytometric analysis of cell cycle

A stain using propidium iodide was used to determine if drug exposure with green tea extract induces cell cycle arrest.²² The HCT-116 cancer cell line was seeded in a 6-well plate at a density of 250,000 cells/

well. After seeding, the cells were allowed 24 hours to fully adhere to the surface of the plate. After attachment, the cells were treated with green tea extract for a 24-hour exposure period. Following drug incubation, the cells were washed with 1X PBS, trypsinized, and centrifuged at 300 g for 5 minutes to pellet. The pelleted cells were then resuspended in 400 µL PBS, and 3 mL of ice-cold 70% ethanol was added dropwise to fix the cell membranes. This solution was then placed on ice for 30 minutes. Next, the cells were centrifuged and resuspended in PBS before adding 0.5 mL of FxCycle (Invitrogen Cat. No. F-10347) to stain. The stain was allowed to incubate at room temperature for 20 minutes before running on an Attune NxT Acoustic Flow Cytometer (Life Tech). Stained cells were analyzed using 488 nm excitation to reveal DNA content within the treated cells.

Results

Green tea extraction and determination of EGCG Content

A green tea extract was made by steeping ground tea leaves in DI water for a 24-hour period. Although an abundance of evidence exists that EGCG is an active ingredient in green tea extracts, we chose to assess the efficacy of extracts, rather than the purified compound because the culturally relevant means of using EGCG is by drinking teas (extracts) containing high concentrations of the compound rather than medically dosing purified material. Further, it remains possible that other ingredients are either independently active or may synergize with EGCG's activity. To normalize dosing on cells we established the concentration of this compound in our extract by HPLC using a commercially available standard and report our findings based on this concentration. In our preparation, we found our stock extract contained 718 mM EGCG.

Cell Viability Assay

The results obtained reveal that green tea extract induces apoptosis selectively in the HeLa cancer cell line (**Figure 1**). Green tea extract displays the highest cytotoxicity on the HeLa cell line at a treatment concentration of 4 mM, which yielded 56% viability. In comparison to the MRC-5 primary cell model, green tea extract shows a minimal cytotoxic effect and cells showed a prolonged resistance to cell death following

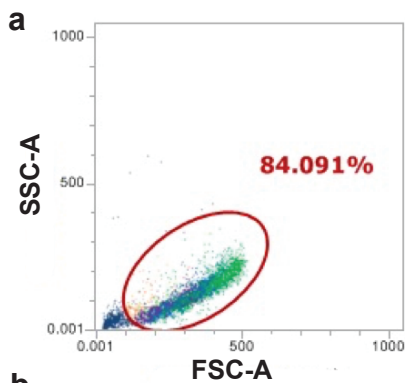
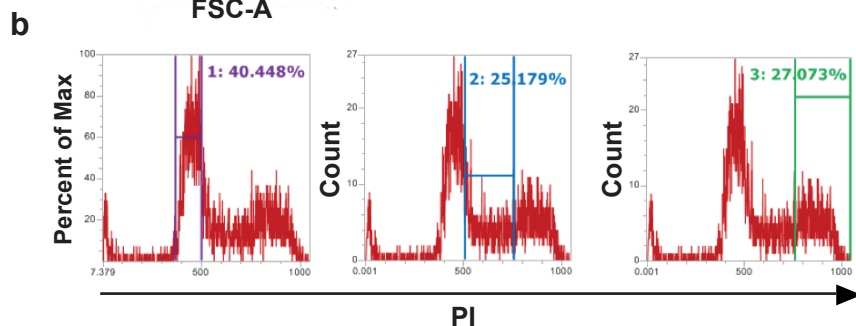


Figure 2 | Cell Cycle Analysis of HCT-116 cells stained with propidium iodide after 24 hours culturing in unsupplemented media. (a) FSC / SSC plot of cells without drug treatment. (b) Histograms of PI-stained cells after 24 hours of culturing without the addition of any extract.

G0/G1	S	G2
40.448	25.179%	27.073



drug exposure. Green tea extract displays preferential cytotoxic effects correlating with cancerous targets. Other cell lines with significant cytotoxic effects include HCT-116 cells, which adhere to the anticancer hypothesis.

Cell Cycle Assessment

To evaluate if green tea extract (normalized for EGCG concentration) induced cell cycle arrest on treated cells, a cell cycle analysis of HCT-116 human colorectal carcinoma cell line was conducted (Figure 2). Green tea extract exposure decreased the percentage of cells in S phase from 25.18% to 20.53%, indicating that treated cells were not dividing their DNA as efficiently as non-treated cells (Figure 3). Correspondingly, cell populations experienced an increased proportion of cells in the G1 phase of the cell cycle from 40.45% to 44.40%. Together, these support a conclusion that Green Tea Extract containing EGCG at 4 mM additionally results in cell cycle arrest in a carcinoma cell line.

Discussion

The hypothesis that green tea's EGCG component plays an anticancer shows potential from the resulting cell viability analysis using MTT. At a concentration of 4 mM, HeLa cells displayed 56% viability post-exposure to green tea extract. This value is comparable to the cell vi-

ability of MRC-5 cells post-exposure to extract, which revealed limited cytotoxic effects. Although a complete kill curve was not observed, drug selectivity is present beginning at 1.4 mM treatment. An increased range including concentrations up to ~8 mM would be necessary to obtain a complete IC50 curve that reveals if drug exposure can cause complete cell death. However, these results indicate a potential for green tea extract to selectively induce cytotoxic effects on cancerous cells while avoiding damage to healthy cells.

In support of green tea's oppressive effect on cancer cells, a cell cycle analysis on the HCT-116 colorectal carcinoma cell line indicated that extract exposure caused cell cycle arrest in the G1 phase. This hypothesis is backed by observation of increased cell population in the G1 phase, in addition to a decrease in cell population in the S phase from treated cells. This reduction in the S phase indicates that fewer cells are dividing their DNA, ultimately resulting in halted cell growth. Not only does green tea extract express a cytotoxic effect on cancerous cells, but it also shows the potential to decrease cell proliferation as well. Though green tea's anti-proliferative effect was not evaluated further, continuing research should analyze how green tea extract affects regulatory mechanisms in the cell cycle.

Cell cycle regulation at the G1 phase is extremely complex, consisting of various cyclins, cyclin-dependent kinases, and cyclin-kinase inhibitors. To evaluate how

green tea extract induces cell cycle arrest, a western blot evaluating protein interactions would be essential to determine what cell regulators are affected.

Future Directions

Future directions include evaluating the mechanisms involved in cell cycle arrest at the G0/G1 phase of cells treated with green tea extract. Cell cycle arrest at this phase indicates that the cells are viable but unable to replicate their DNA in the S phase resulting in decreased proliferation. As Cyclin E and CDK2 play key roles in regulating this checkpoint, the interaction between these and green tea should be examined to determine their relationship. Suppression of proliferation is a promising aspect of utilizing green tea as a therapeutic for cancer cells and opens the possibility for EGCG to be considered as a potential cancer preventative. Though the EGCG component was not specifically isolated from green tea extract, there is a promise of the potential inhibition of regulatory cyclins through green tea's EGCG component.

Acknowledgments

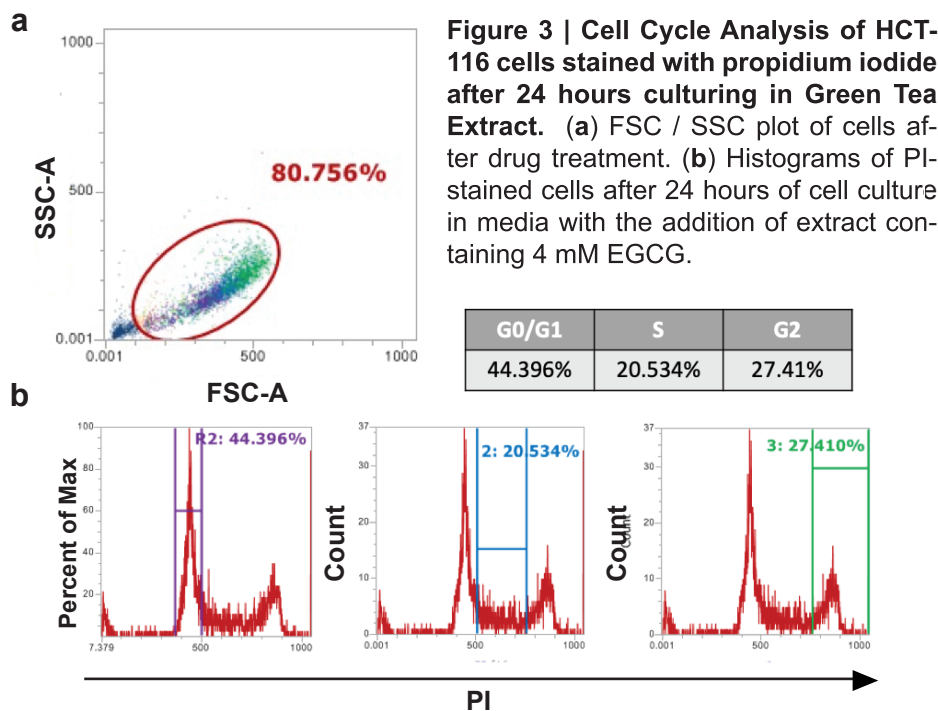
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Author's Biography

Allayah Stillwell is a senior in the KU Biotechnology program and will be continuing research as an intern at Genentech in Early Clinical Development. With her interest in drug development and oncology, she plans to pursue medical school in the upcoming year.

Author Contributions

A.A.S. contributed to the experimental work, design, and writing of this work; J.F.T. contributed to the editing and design of this work; R.L.L. contributed to the editing and design of this work.



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