Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays

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Black tea extracts (hot aqueous, polyphenols and theaflavins) and green tea extracts (hot aqueous, polyphenols, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate) were tested in nine standardized cell culture assays for comparative cancer chemopreventive properties. Most black and green tea extracts strongly inhibited neoplastic transformation in mouse mammary organ cultures, rat tracheal epithelial cells and human lung tumor epithelial cells. Nearly all tea fractions strongly inhibited benz[a]pyrene adduct formation with human DNA. Induction of phase II enzymes, glutathione-S-transferase and quinone reductase, were enhanced by nearly all tea fractions, while glutathione was induced by only a few fractions.

Introduction

Next to water, tea is the most ancient and widely consumed beverage in the world. Three types of tea are predominately manufactured: black tea, green tea and oolong tea. Epidemiologic studies have suggested a cancer protective effect, but this result is not conclusive, since both positive and negative associations have been observed. Reductions in human cancer of the breast (1), colon and rectum (2–4), gall bladder (5), liver (1), lung (6–8), nasopharynx (9), pancreas (10), stomach (3,9,11) and uterus (7) have been reported associated with green tea consumption. However, increased cancer incidences have been cited for breast, colorectal, esophageal, kidney, lung, pancreas and stomach (3). These inconsistencies may be attributable to the consumption of hot or salted tea, influence of lifestyle habits such as drinking or smoking, or to geographical location.

Most experimental animal studies show that tea has cancer preventive properties and these studies have been reviewed extensively in recent years (12–19). For example, Wang et al. (20) showed that green tea extract (GTE) inhibited N-nitrosodiethylamine-induced forestomach and lung tumors in mice and that both GTE and black tea extract (BTE) inhibited 4-(methylnitrosamino)-1-butanone-induced lung adenomas, also in mice. Recently Rodgers et al. (21) showed black tea consumption could decrease mammary cancer in rats on high fat diets.

There has been extensive research into the possible mechanisms of cancer prevention by tea extracts. Such mechanistic studies, especially with green tea polyphenols (GTPs) and epigallocatechin gallate (EGCG), include inhibition of free radical formation and lipid peroxidation (22–31), the inhibition of ornithine decarboxylase (ODC) (32–34), and the inhibition of DNA–carcinogen binding and adduct formation (32,35). In addition there have been reports of the inhibition of cyclooxygenase and lipoxygenase activity by tea compounds (32,36) and inhibition of protein kinase C and cellular proliferation (34,37–39). Laio and Hiipakka (40) reported that certain green tea catechins can inhibit 5α steroid reductase isoenzymes. They showed inhibition by (−)-EGCG and epicatechin gallate (ECG), but not epicatechin (EC) or epigallocatechin (EGC). EGCG and ECG were more potent inhibitors of type I than type II reductase. These results are supported by the further findings of Laio et al. (41) which showed that human prostate and breast tumor cell growth was inhibited by EGCG, but not other catechins. In an in vitro assay using solubilized prostate microsomes, we have observed a 75% inhibition of 5α reductase activity by EGCG with an IC 50 of 51 μM (or an ED 50 of 30 μM) (unpublished data).

The studies presented here are the first direct comparative associations have been observed. Reductions in human cancer of the breast (1), colon and rectum (2–4), gall bladder (5), liver (1), lung (6–8), nasopharynx (9), pancreas (10), stomach (3,9,11) and uterus (7) have been reported associated with green tea consumption. However, increased cancer incidences

Abbreviations: B(a)P, benzo[a]pyrene; BTE, black tea extract; BTP, black tea polyphenols; DMBA, 7,12-dimethylbenz[a]anthracene; DMPO, difluoro-methylornithine; DMSO, dimethyl sulfoxide; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; ETOAc, ethyl acetate; GSH, glutathione; GST, glutathione-S-transferase; GTE, green tea extract; GTP, green tea polyphenols; MMOC, mouse mammary organ culture; NADPH:QR, quinone reductase; ODC, ornithine decarboxylase; RTE, rat tracheal epithelial; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Materials and methods

BTEs and GTEs, polyphenol fractions and purified polyphenols were kindly provided by the T.J.Lipton Tea Company (Englewood Cliffs, NJ). The GTEs and BTEs were prepared from standard tea mixtures and were characterized as follows. BTE, caffeine-free: freeze-dried hot water extract, 44.94% polyphenols,
2.95% EGCG, 2.15% theaflavins, 5.00% caffeine, 44.96% unknown; BTE, decaffeinated: freeze-dried hot water extract, 41.90% polyphenols, 2.67% EGCG, 3.39% theaflavins, <0.1% caffeine, 52.04% unknown; GTE, caffeine: freeze-dried hot water extract, 44.72% polyphenols, 10.33% EGCG, <0.1% caffeine, 44.95% unknown; black tea polyphenol (BTP): <3% moisture, 1.06% caffeine, <100 p.p.m. ethyl acetate (EtOAc),<5 p.p.m. methylene chloride, 3% gallic acid, 2% catechin, 7% EC, 2% EGC, 13% ECG, 17% EGCG, 2% other non-ECs, 5% flavonol glycosides, 8% theaflavins, 32% unknown polyphenols; green tea polyphenol (GTP): <3% moisture, 82% caffeine, <100 p.p.m. EtOAc,<5 p.p.m. methylene chloride, 1% catechin, 10% EC, 15% EGC, 10% ECG, 43% EGCG, 2% other non-ECs, 1% flavonol glycosides, 13% other polyphenols; theaflavin mixture: 7.65% theaflavins, 30.59% theaflavin gallate A, 19.45% theaflavin gallate B, 32.17% theaflavin digallate, 4% water, 6.14% unknown; EC: MW 290.28, purity 98%: EGC: MW 306.28, purity 95%; ECG: MW 442.28, purity 91.67%; EGCG: MW 458.28, purity 92.5%.

In vitro assay procedures

Inhibition of carcinogen binding. This assay tests the ability of an agent to prevent benzo[a]pyrene (BaP)-diol epoxide from forming a stable complex with DNA (43). In this assay, the level of DNA binding to immortalized human bronchial epithelial cells, the BEAS-2B cell line, is measured by detecting the amount of DNA-bound [3H]-labeled BaP. Results are calculated as percent reduction of carcinogen-DNA binding, compared with control. An agent is considered positive if one or more non-toxic concentrations inhibit DNA binding by at least 15%.

Glutathione (GSH) induction. This assay identifies chemopreventive agents which increase the ability to induce GSH in normal human Chang liver cells (44). GSH levels are measured by a fluorometric method following exposure to test agents at five non-toxic concentrations. Results are calculated as percent increase in GSH compared with vehicle controls. An agent is considered positive if it induces GSH by at least 10% at one or more non-toxic concentrations.

ODC inhibition. This test identifies potential chemopreventive agents which are able to inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ODC in 2C5 cells (45). The assay is based on quantification of [3H]Hpturespicine, a product of ODC-catalyzed decarboxylation of [3H]Hornithine. Historically, the mean inhibition by difluoromethylmethionine (DFMO; the positive control) is 57 ± 18%. An inhibition of >15% at one or more non-toxic concentrations is considered a positive result.

NADPH:QR activity. Human liver cells (Chang) are used to identify agents with ability to induce QR using a microtitr plate assay (46,47). Results are expressed as percent QR increase over controls. QR induction ≥25% by the test agent, compared with the solvent controls is considered a positive result.

GST induction. The GST induction assay is based on the conjugation rate of 1-chloro-2,4-dinitrobenzene with GSH which can be followed spectrophotometrically at 340 nm (48). Agents are then added to the cells at five concentrations (1 mM or the next highest soluble concentration plus four log). Positive and negative controls are oltipraz (50 µM) and the appropriate solvent, respectively. Specific GST activity is calculated and compared with the activity of the solvent control. Results which demonstrate at least 15% induction compared with the solvent control are considered positive.

Free radical scavengers/antioxidants. Free radical generation induced by TPA in human HL-60 cells is measured by detecting the amount of DNA-bound 3H-labeled BaP (49). Results are calculated as the percent decrease in oxygen radical formation for test agent plus TPA, compared with TPA treatment alone. Results which demonstrate an induction of 10% in at least one concentration are considered positive.

Human tumor cell (A427) anchorage-independent growth inhibition assay. The established human lung tumor cell line, A427, is used (50). The expression of the anchorage-independent phenotype is measured by the ability of the cells to form colonies in semi-solid agarose medium. Modulation of the anchorage-independent phenotype is determined by the addition of the chemopreventive agent to a semi-solid medium at the time of cell seeding. After ~28 days, the dishes are stained using a tetrazolium salt and the colonies counted. Agents were scored positive if they inhibit anchorage-independent growth by 20% or more at one or more test concentrations, compared with solvent control cultures.

The rat tracheal epithelial (RTE) focus inhibition assay. The assay used is identical to that published previously (51). Tracheal epithelial cells are isolated from 8–12-week-old male Fisher 344 rats and plated on collagen coated dishes. The maximal non-toxic concentration and four half-log lower concentrations are used for the chemopreventive test assays. At day 30 all cultures were fixed, stained and morphologically altered foci are scored. The transformation frequency of the various groups is determined. The percent reduction (induced by the chemopreventive test agent) of transformation frequency (BaP alone
group) was calculated for each dose level. Agents were considered positive if they inhibit anchorage-independent growth by 20% or more with vehicle controls at two or more concentrations.

7,12-Dimethylbenz[a]anthracene (DMBA)-induced mouse mammary organ culture (MMOC) assay. The procedure for the induction of DMBA-induced mammary lesions has been described in detail previously (52). The chemopreventive agents incorporated in the medium for the first 10 days of culture at five different concentrations ranging from 10⁻⁹ to 10⁻³ M. At the end of the 24 day experiment, the glands are fixed in formalin, stained with alun carmine, and evaluated for the incidence and multiplicity of the mammary lesions in these glands. Agents were considered positive if they inhibit lesions in >60% of the glands or a x² analysis shows statistically significant inhibition (P < 0.05), compared with the DMBA control group.

Results

The black and green tea extracts were assayed for activity in three in vitro cell transformation assays: the RTE, A427 and MMOC assays (Table I). In the RTE assay, BTE inhibited transformation by 22–37% at concentrations ranging from 0.1 to 10 ng/ml. On the other hand, the decaffeinated BTE inhibited RTE transformation by 86–100% at 0.01–1 ng/ml. When BTP was added, transformation was inhibited to a similar extent but at higher concentrations (3–300 ng/ml). The theaflavin mixture (1 ng/ml) was also very effective. GTE inhibited transformation from 70 to 83% at concentrations ranging from 3–300 ng/ml. The decaffeinated GTE had a somewhat lower (47–53%) inhibition at comparable doses. GTP also had a high percentage of inhibition (74–100%) at similar doses. Two of the four tea catechins, ECG and EGCG, were also effective.

Therefore, both black and green tea extracts or polyphenols were very effective at inhibiting BaP-induced RTE transformation.

The same fractions were tested in the human lung tumor cell (A427) assay for the inhibition of anchorage-independent growth. BTE inhibited anchorage-independent growth by 50–90% at concentrations of ~4–125 µg/ml, while BTP inhibited anchorage-independent growth by 90–100% at similar concentrations. Decaffeinated BTE was more inhibitory (82–93%) than BTE. The theaflavins, GTE and decaffeinated GTE showed little inhibition, while GTP were quite effective (74–92% inhibition). All the green tea catechins were effective in inhibiting anchorage-independent growth of A427 cells.

In the MMOC assay, the two BTEs (BTE and decaffeinated BTE) were quite effective. Surprisingly, the polyphenol and

<table>
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<tr>
<th>Table I. Activity of tea compounds in in vitro cell transformation assays</th>
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<td>Agents</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>A427</td>
</tr>
<tr>
<td>Black tea, decaffeinated</td>
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<tr>
<td>Black tea, caffeinated</td>
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<tr>
<td>Black tea polyphenol</td>
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<tr>
<td>Theaflavins</td>
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<tr>
<td>Green tea, decaffeinated</td>
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<tr>
<td>Green tea, caffeinated</td>
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<tr>
<td>Green tea polyphenols</td>
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<tr>
<td>Epicatechin</td>
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<td>Epicatechin gallate</td>
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<tr>
<td>Epigallocatechin</td>
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<td>EGCG</td>
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A427, human cell line anchorage-independent growth inhibition; RTE, rat tracheal epithelial transformation inhibition; MMOC, mouse mammary organ culture alveolar nodule inhibition; +, effective (IC₅₀ in µg/ml); NE, not effective.
Theaflavin mixtures were ineffective in inhibiting DMBA-induced mammary gland transformation. Green tea extracts were largely ineffective, although decaffeinated GTE, GTP and EGCG were effective.

A series of six biochemical assays were also performed using these same compounds and mixtures (Table II). No extracts were positive in all six assays, but several were positive in four to five assays, notably EGC, ECG, GTP, GTE and BTP. In the assay which measures inhibition of \( B[a]P \) binding to human epithelial cell DNA, all tea fractions except green tea (caffeinated) were highly active (Table II). The tea extracts were tested at concentrations from 0.01 to 100 \( \mu \)g/ml. BTE inhibited \( B[a]P \) binding by ~60–80% at nearly all concentrations. The BTP were equally effective at the same concentrations. The theaflavin mixture inhibited binding from ~20–80% at the above concentrations. On the other hand GTE tested at 0.1–1000 \( \mu \)g/ml had no inhibitory effect in this assay. GTP gave up to ~40% inhibition at lower concentrations. The purified tea catechins were all highly effective when tested at 0.0001–1 mM. EC inhibited \( B[a]P \) binding by ~60%, while ECG inhibited up to 50% of the binding. EGC inhibited 100% at the highest dose and 30–40% at lower doses. EGCG inhibited binding at 40–75% at similar concentrations.

Fewer tea agents were active in increasing GSH content. BTE and theaflavins caused <10% induction of GSH. BTP induced GSH by 11% maximally, and were given a ‘+’ score. GTE induced GSH by 15–27% over controls, but GTP were inactive. None of the four purified polyphenol fractions (EGCG, EC, EGC and EGCG) was effective in inducing GSH in this assay.

The inhibition of ODC activity distinguished the black and green teas when they were tested at 0.0001–1 mg/ml (or mM for the purified extracts). Black teas were ineffective as a whole, while both GTE and three of the four purified extracts were positive. GTE inhibited ODC from 80 to 98% at all the concentrations tested. EC inhibited ODC by ~40%, while EGC inhibited ODC by ~80% at all concentrations. GTP and ECG showed minimal activity. EGCG was ineffective in the ODC inhibition assay.

In a similar pattern NADPH:QR was induced primarily by GTE and not BTE when tested at concentrations ranging from 0.0001 to 1 mg/ml (or mM for the purified extracts). Only BTP induced QR by 40–70%, while the BTE and theaflavins were negative. The two GTE and GTP maximally induced QR by 40 and 60%, respectively. EC, EGC and ECG induced by 60–70%, while EGCG was negative.

The induction of GST by BTE and GTE was ~100 and 10%, respectively, while induction by both polyphenol fractions was ~30–40%. The concentrations used were 0.0001–1 mg/ml (or mM for the purified extracts). Theaflavins showed no activity inducing GST. ECG, EGC and EGCG were all inducers of GST (40–60%), while EC had little effect.

Both BTE and GTE inhibited free radical formation when tested at concentrations similar to the GST test above. The tea extracts and theaflavins inhibited radical formation by ~60%, while BTP gave marginal results. Of the four purified tea extracts only ECG and EGC were positive, inhibiting radical formation by ~20–30%.

**Discussion**

The results presented here, as well as past literature, show that tea extracts have many possible chemopreventive mechanisms. Tea compounds also can act by decreasing xenobiotic metabolism, decreasing nitrosification reactions and modulating immune function. These activities could explain the inhibition of B[a]P binding to DNA which was seen in the present studies, and could explain the inhibition of polycyclic aromatic hydrocarbon-induced mouse skin cancer (34) and the marked inhibition of B[a]P-induced transformation of RTE cells.

GSH is a natural antioxidant found in the diet, and can protect the cell during both the initiation and the promotion phases of carcinogenesis. GSH conjugation is involved in the reaction of electrophiles, such as reactive metabolites of B[a]P, with the nucleophilic thiol moiety of GSH. Such a mechanism would decrease the level of reactive electrophiles available to bind to DNA, reducing the likelihood of the DNA damage and possible induction of the carcinogenesis process. GSH could also protect the cells by limiting oxidative free radical attack. However, GSH was only induced by three of the nine tea extracts and, therefore, is probably not a strong candidate for tea’s cancer inhibitory activity.

ODC activity is stimulated in mouse epidermal cells following application of known tumor promoting agents. Diacetylcysteine (the putative ligand of protein kinase C) and TPA-treated RTE cells (2C5 cell line) show increased levels of ODC (53). This induction can be blocked by the addition of retinoic acid. The inhibition of polyamine synthesis could possibly block or slow down the promotion phase of carcinogenesis. It is extremely interesting that only GTE and not BTE inhibited ODC activity. Further work should be done to determine the causative factors important to this unique finding.

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**Table II. Effect of tea extracts on chemoprevention-related biochemical endpoints**

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<tr>
<th>Agent</th>
<th>Prescreening biochemical assays</th>
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<tr>
<td></td>
<td>( B[a]P )</td>
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<tr>
<td>Black tea, decaffeinated</td>
<td>+++++</td>
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<tr>
<td>Black tea polyphenols</td>
<td>+++++</td>
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<tr>
<td>Theaflavins</td>
<td>+++++</td>
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<tr>
<td>Green tea, decaffeinated</td>
<td>NE</td>
</tr>
<tr>
<td>Green tea polyphenols</td>
<td>+++</td>
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<tr>
<td>Epicatechin</td>
<td>+++</td>
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<tr>
<td>Epicatechin gallate</td>
<td>+++</td>
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<tr>
<td>Epigallocatechin</td>
<td>+++</td>
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<tr>
<td>EGCG</td>
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</table>


Relative activity: +, low; +++, moderate; +++++, high.
NADPH:QR is an electrophile processing metabolic enzyme. It is widely distributed, is primarily cytosolic and catalyzes the reduction of a wide variety of quinones. QR is induced coordinately with phase II metabolizing enzymes by a variety of compounds that protect rodents from the toxic, mutagenic and neoplastic effects of carcinogens (54). In the current study, BTE was more effective in inducing GST (100%) than QR (21%). Since 25% induction was used as a criterion for effectiveness, BTE was considered negative. In addition, significant toxicity was noticed at higher concentrations. There is a large body of evidence suggesting that monitoring these enzymes is a convenient screening method for anti-carcinogenic activity (47).

GSTs are a family of phase II isoenzymes that catalyze the conjugation of GSH to a variety of electrophilic compounds, including carcinogens and cytotoxic drugs. These enzymes probably form part of an inherent protective mechanism against the development of tumors, which may be caused by chemical carcinogens present in the environment. The detoxification of B[a]P in the RTE assay and DMBA in the MOC assay by enhanced QR and/or GST activity could contribute to the preventive activity of many of the tea extracts.

Oxygen and other free radicals can be induced by a variety of methods in the cell. These radicals pose a potential threat to the genome by acting directly on nuclear chromatin, by interfering with the DNA replication/repair machinery of the cell, or through an indirect mechanism such as lipid peroxidation. TPA can produce oxygen radicals and peroxides in lymphocytes. Inhibition of free radical formation by many of the tea extracts was seen and could be a primary mechanism by which tea extracts prevent carcinogenesis. A primary culture system using RTE cells has been developed to quantitatively determine the efficacy of chemopreventive agents in inhibiting the process of neoplastic transformation in lung (55). In this system, primary cultures of RTE cells are exposed to carcinogens on day 1, then morphologically altered epithelial foci are scored at day 30. Daughter cells from these carcinogen-induced foci have been shown to produce malignant carcinomas when injected into nude mice (56). Many of the tea extracts prevented RTE transformation and are now in testing to prevent pulmonary cancer in whole animals.

There is limited pharmacokinetic data available for uptake and distribution of tea compounds in experimental animals (57). Administering 0.9% GTE (decaffeinated) to rats for 3 weeks, plasma levels of EGCG were at ~0.08 µM. These achievable and non-toxic plasma levels were far above those which showed efficacy in the RTE and A427 assays cell culture (0.003 and 0.005 µM, respectively). However the concentration showing efficacy in the MOC assay (44 µM) is much higher than achievable plasma levels. Recent work by Hirose et al. (58) has concluded that EGCG-rich polyphenon E showed little effect on DMBA-induced rat mammary cancer. Where green tea has been shown to inhibit cancers in whole animals, the dosages given are at or below 0.9% and within the range used in the A427 and RTE studies (12).

Several chemopreventive agents, including retinoids, selenium, β-carotene, etc., have been tested for their inhibitory activity against DMBA-induced hyperplastic alveolar nodule-like mammary lesions in organ cultures (59–62). There appears to be a good correlation between the inhibition of DMBA-induced lesion formation in vitro and their efficacy against mammary carcinogenesis in vivo (63).

In summary, BTE, either caffeinated and decaffeinated, were highly effective inhibitors of transformation in all three in vitro assays, while similar GTE were not quite as effective. The BTP and GTP fractions appeared quite active in most assays. The theaflavin fraction of black tea and the catechin fractions of green tea showed mixed responses. Almost all tea fractions inhibited carcinogen binding to DNA in human cells. Few tea fractions induced GSH and only green tea fractions inhibited ODC. A majority of tea extracts induced QR and phase II enzymes, such as GST. Most tea fractions inhibited free radical formation in human cells. As measured by these nine in vitro assays, the data suggest (i) that tea compounds can inhibit the transformation process at many checkpoints and (ii) that both black and green tea extracts may be equally effective in preventing cancer in humans.

References
Chemoprevention by tea extracts


