The Antifolate Activity of Tea Catechins

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Abstract

A naturally occurring gallated polyphenol isolated from green tea leaves, (−)-epigallocatechin gallate (EGCG), has been shown to be an inhibitor of dihydrofolate reductase (DHFR) activity in vitro at concentrations found in the serum and tissues of green tea drinkers (0.1–1.0 μmol/L). These data provide the first evidence that the prophylactic effect of green tea drinking on certain forms of cancer, suggested by epidemiologic studies, is due to the inhibition of DHFR by EGCG and could also explain why tea extracts have been traditionally used in “alternative medicine” as anticarcinogenic/antibiotic agents or in the treatment of conditions such as psoriasis. EGCG exhibited kinetic characteristics of a slow, tight-binding inhibitor of 7,8-dihydrofolate reductase with bovine liver DHFR (Ki = 0.109 μmol/L), but of a classic, reversible, competitive inhibitor with chicken liver DHFR (Ki = 10.3 μmol/L). Structural modeling showed that EGCG can bind to human DHFR at the same site and in a similar orientation to that observed for some structurally characterized DHFR inhibitor complexes. The responses of lymphoma cells to EGCG and known antifolates were similar, that is, a dose-dependent inhibition of cell growth (IC50 = 20 μmol/L for EGCG), G0-G1 phase arrest of the cell cycle, and induction of apoptosis. Folate depletion increased the sensitivity of these cell lines to antifolates and EGCG. These effects were attenuated by growing the cells in a medium containing hypoxanthine-thymidine, consistent with DHFR being the site of action for EGCG.

Introduction

Green tea catechins that include (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) exhibit a range of biological activities (1–3). EGCG has been the most extensively studied because of its relatively high abundance and strong epidemiologic evidence for cancer prevention (4). EGCG have been shown in vitro to stimulate apoptosis and cell cycle arrest of various cancer cell lines, including prostate, lymphoma, colon, and lung (1). The site of action and mechanism at the molecular level by which EGCG acts as an anticarcinogen is poorly understood. EGCG has been implicated in the modulation of several transcription factors such as activator protein 1 and nuclear factor κB, inhibition of gene expression such as tumor necrosis factor α, vascular endothelial growth factor, and nitric oxide synthase, and modulation of several cancer-related proteins that include urokinase, ornithine decarboxylase, matrix metalloproteinase, and cyclooxygenase (see ref. 4 and references therein). In addition, ester bond–containing tea polyphenols potently inhibit proteasome activity (5). EGCG binds strongly to many biological molecules and affects a variety of enzyme activities and signal transduction pathways at concentrations from milli- to nanomolar (6). The effective concentration of EGCG in the blood or tissues of tea drinkers is in the range 0.1 to 1.0 μmol/L (6), an important factor in deciding whether an in vitro modulation of biological activity by EGCG is likely to be relevant in vivo.

In attempting to explain the range of responses of normal and cancer cells to tea phenols observed in our laboratory and those of others, we were intrigued by the structural similarity of EGCG to several inhibitors of dihydrofolate reductase (DHFR), in particular the drugs methotrexate (Fig. 1) and aminopterin. DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate, which acts as a coenzyme for several one-carbon group transfer reactions that include steps in nucleotide biosynthesis. Consequently, inhibition of DHFR, resulting in the disruption of DNA biosynthesis, is the basis of the chemotherapeutic action of a range of DHFR inhibitors, generically known as “antifolates.” Tumor cells that grow rapidly require a higher concentration of dITTP than normal cells, and therefore are more sensitive to antifolates. We have therefore tested our hypothesis that the differential physiologic effects of tea polyphenols on normal and cancer cell lines can be explained if they have pronounced “antifolate activity” by studying in vitro the inhibition of DHFR isolated from two sources, bovine and chicken livers. We have also used the published X-ray structure of human DHFR bound to a tetrahydrodiquinoxaline inhibitor, (R)-6-[[(3,4,5-trimethoxyphenyl)amino]methyl]-5,6,7,8-tetrahydrodiquinoxaline-2,4-diamine (TQD; Fig. 1) to model the binding of EGCG in a manner that can explain the observed tight binding and competitive inhibition with respect to DHF.

Materials and Methods

Dihydrofolate Reductase Assay and Kinetic Data Analysis. Activity measurements for DHFR isolated from chicken liver (Sigma, Madrid, Spain) and bovine liver (Fluka, Madrid, Spain) were made by following the decrease of NADPH (Sigma) and DHF (Sigma) absorbance at 340 nm (Δε = 11,800 mol L−1 cm−1) using a Perkin-Elmer Lambda-2 spectrophotometer thermostatted at 25°C with 1.0-cm path-length cuvettes. Experiments were done in a buffer containing MES (0.025 mol/L), sodium acetate (0.025 mol/L), Tris (0.05 mol/L), and NaCl (0.1 mol/L). To prevent the oxidation of catechins (EGCG, EGC, ECG, and EC purchased from Sigma) the reaction mixture contained 1 mmol/L ascorbic acid (Scharlau, Barcelona, Spain). Initial velocity inhibition experiments were done at a constant and saturating concentration of NADPH (100 μmol/L), whereas concentrations of DHF and the inhibitors (catechins) varied from 0 to 20 μmol/L and from 0 to 100 μmol/L, respectively.
The action of folate analogues, which act as slow-binding inhibitors \((I)\) on DHFR \((E)\), can be described by the following mechanism:

\[
E \xrightleftharpoons[k_1]{k_2} EI \xrightleftharpoons[k_3]{k_4} EI^* \quad (A)
\]

Although the DHFR-catalyzed reaction has been shown to occur via a random mechanism \((7)\), it can be simplified to an ordered mechanism whenever \([\text{NADPH}] \gg [\text{DHF}]\). If the concentration of free inhibitor is not substantially altered by the formation of an enzyme-NADPH inhibitor complex, the progress curve for the inhibition in the presence of saturating NADPH can be described by Eq. B:

\[
P = v_s t + \left( v_0 - v_s \right) \left( 1 - \exp\left(-k't\right) \right)/k' \quad (B)
\]

where \(v_s\), \(v_0\), and \(k'\) represent the steady-state velocity, initial velocity, and apparent first-order rate constant, respectively. The apparent first-order rate constant is related to the inhibitor concentration by Eq. C, where \(K_m^\text{DHF}\) is the Michaelis constant of DHFR for DHF:

\[
k' = k_4 \left[ \frac{1 + \frac{[I]}{K_I}}{1 + \frac{[I]}{K_I^*}} \right] \quad (C)
\]

\(K_I\) and \(K_I^*\) denote the respective dissociation constants for the initial and equilibrium binding of inhibitors to the enzyme-NADPH complex. The slow development of catechin inhibition was determined by continuously monitoring the disappearance of NADPH and DHF after initiation of the reaction by the addition of DHFR \((3.3 \text{ nmol/L})\). Reaction mixtures contained buffer mixture, NADPH \((100 \text{ mM})\), DHF \((20 \text{ mM})\), and various concentrations of catechins from 0 to 20 \text{ mM}. Experiments to determine the maximum steady-state rate \((v_s)\) and \(K_m^\text{DHF}\) at several pH values required the analysis of the curvature evident in the time courses for the disappearance of NADPH and DHF \((10\) determinations). The initial concentration of saturating NADPH \((200 \text{ mM})\) was considered to be constant over the period required for the consumption of 10 \text{ mM} DHF after the addition of DHFR \((6 \text{ nmol/L})\). Data were fitted by nonlinear regression to the integrated form of the Michaelis equation \((8)\). The extent of recovery of enzymatic activity after inhibition induced by preincubation with catechins was determined as follows. DHFR \((165 \text{ nmol/L})\) was preincubated for 30 minutes at 25°C in the buffer mixture (pH 8.02).

\[
K_I^* = \frac{K_I k_3}{k_3 + k_4} \quad (D)
\]

\[
K_I = \frac{k_2}{k_1} \quad (E)
\]

Figure 1. A, structural formulas of \((-\text{)}\)-epigallocatechin gallate, TQD, and methotrexate. B, wall-eyed stereo view of EGCG modeled into the folate-binding site of human DHFR. Carbon atoms of the EGCG ligand and surrounding protein are colored green and gray, respectively. Residue Phe\(^{31}\), located behind the EGCG, is unlabeled. Four different ligands from human and chicken DHFR crystal structures were used to define a binding envelope, shown in cyan; these were placed in a common orientation by superimposing backbone atoms from a common set of protein residues located around the ligands. Ligands from the following Protein Data Bank structure files were used: 1DR1 (bioppterin), 1S3V (TQD), 1S3W, and 1DLR.
ascorbic acid. With the folate-depleted experiments, IC_{50} values of EGCG were compared with values of untreated control cells. For the time course experiments, cells were plated as described above and treated with 20 µmol/L EGCG concentration that gave a 50% decrease in cellular growth compared with values of untreated control cells. For the time course study, cells were plated as described above and treated with 20 µmol/L EGCG. Reversion experiments were done in a medium containing assay mixtures. Such behavior can be described by a mechanism that involves the rapid binding of the inhibitor (EGCG) to the enzyme (DHFR) to form an EI complex which then undergoes a slow isomerization to form an E{\textsuperscript{r}} complex (Eq. A). Such a mechanism of inhibition of DHFR has been previously reported for folate analogues such as methotrexate and deazaflavines (7, 12). A complete kinetic analysis of the inhibition of bovine liver DHFR yielded the kinetic parameters given in Table 1. Although ECG was also a potent inhibitor, polyphenols lacking the

**Results and Discussion**

**Inhibition of Dihydrofolate Reductase by Tea Catechins.** Steady-state kinetic data showing the inhibition by EGCG of DHF reduction with chicken liver DHFR are shown in Fig. 2A. A K_{1} (10.3 µmol/L) for EGCG as a competitive inhibitor of DHF calculated from the secondary plot (Fig. 2B) is compared in Table 1 with values for methotrexate (1.3 µmol/L) and trimethoprim (3.5 µmol/L). Preincubation of the enzyme with EGCG did not produce any measurable inhibition. Thus, the inhibition shown in Fig. 2A and B must involve rapid reversible binding of EGCG to chicken liver DHFR. However, EGCG acted as a slow-tight-binding inhibitor of bovine liver DHFR (Fig. 2C). In the absence of EGCG, the steady-state velocity of DHF reduction is rapidly established and only shows a minor deviation from linearity over a 15-minute period due to substrate (DHF) depletion. However, in the presence of EGCG, a time-dependent decrease in the reaction rate, which varies as a function of the inhibitor concentration, is clearly apparent in Fig. 2C and D. Further evidence for slow-binding inhibition was obtained by adding aliquots of preincubation mixtures of EGCG and the bovine liver enzyme to substrate-containing assay mixtures. Such behavior can be described by a mechanism that involves the rapid binding of the inhibitor (EGCG) to the enzyme (DHFR) to form an EI complex which then undergoes a slow isomerization to form an E{\textsuperscript{r}} complex (Eq. A).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Inhibition of chicken liver (A and B) and bovine liver (C and D) dihydrofolate reductases by EGCG. **A.** Lineweaver-Burk plots of the reaction of chicken liver DHFR with DHF and NADPH, EGCG concentrations were (•) 0, (○) 25, (■) 50, and (△) 100 µmol/L. Points, mean of five separate experiments; bars, SD. **B.** secondary plots for the apparent Michaelis constant of DHFR for dihydrofolate (K_{m}^{DHF}), obtained from Fig. 1A, versus the concentration of EGCG. **C.** progress curves for the slow, tight-binding inhibition of bovine liver DHFR by EGCG. **D.** nonlinear regression analysis of the progress curves presented in Fig. 1C to Eq. A yield the values of k' at different EGCG concentrations. Points, mean of triplicate determinations; bars, SD.
Table 1. Comparison of the inhibition by methotrexate, trimethoprim, and EGCG of dihydrofolate reductase activity

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Inhibitor</th>
<th>$K_i$ (nmol/L)</th>
<th>$k_3$ (min$^{-1}$)</th>
<th>$k_4$ (min$^{-1}$)</th>
<th>$k_3k_4$ (ratio)</th>
<th>$K_i^*$ (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine liver</td>
<td>EGCG</td>
<td>109</td>
<td>0.13</td>
<td>0.004</td>
<td>32.5</td>
<td>3,253</td>
</tr>
<tr>
<td></td>
<td>ECG</td>
<td>51.3</td>
<td>0.11</td>
<td>0.015</td>
<td>7.3</td>
<td>6,156</td>
</tr>
<tr>
<td>Chicken liver</td>
<td>EGCG</td>
<td>10,300</td>
<td>1.3</td>
<td>2.9</td>
<td>0.020</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>3,530</td>
<td></td>
<td></td>
<td></td>
<td>8.9</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Methotrexate</td>
<td>3.6</td>
<td>6.9</td>
<td>0.026</td>
<td>265.4</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>0.49</td>
<td>2.0</td>
<td>0.086</td>
<td>23.3</td>
<td>20.2</td>
</tr>
<tr>
<td>Streptococcus faecium</td>
<td>Methotrexate</td>
<td>23</td>
<td>5.1</td>
<td>0.013</td>
<td>392.3</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>4.6</td>
<td>2.1</td>
<td>0.58</td>
<td>3.6</td>
<td>1,000</td>
</tr>
</tbody>
</table>

NOTE: Values for the inhibition of DHFR from different sources by methotrexate and trimethoprim were obtained from the literature (7). The values of $K_i$, $K_i^*$, $k_3$, and $k_4$ for EGCG were calculated at pH 8.02.

Ester bonded gallate moiety (i.e., EGC and EC) did not inhibit bovine DHFR activity. These results indicate that the ester bonded gallate moiety is essential for potent inhibition of bovine liver DHFR. Furthermore, a green tea extract containing significant amounts of EGCG also strongly inhibited the DHFR activity of both the bovine and chicken liver enzymes. Methotrexate is a stronger inhibitor ($K_i^*$, picomolar range) than EGCG ($K_i^*$, nanomolar range) for the two DHFRs studied. EGCG should therefore be assigned to the class of “soft” DHF analogue inhibitors of DHFR (13). Such compounds have several advantages in the treatment and prevention of cancer because they can attenuate the adverse side effects often associated with DHFR inhibitors such as methotrexate that are currently in clinical use.

Further evidence for EGCG binding to the same site on DHFR as methotrexate comes from the pH dependence of the $K_i^*$ for the bovine liver enzyme. A plot of $V / K_m^{	ext{DHF}}$ values against pH yielded a single $pK_a$ value of 8.7 ± 0.2, which is similar to that reported for the human enzyme (12). This has been interpreted in the case of the human enzyme as a shift of the $pK_a$ of Glu$^{30}$ from an intrinsic value of 5.6 to an observed value of about 8.7 on DHF binding. The similarity of the two pH profiles for the bovine and human enzymes suggests that the same ionizing residue is involved in catalysis. Because Glu$^{30}$ is the only acidic residue at the pterin subsite of both enzymes (12, 14), it is considered to be the source of the proton for the reduction of DHF (12). The pH dependence of $K_i^*$ for EGCG binding to bovine DHFR gave a $pK_a$ value of 6.5 ± 0.12, which we assign to the Glu$^{30}$ that is conserved in mammalian DHFRs. This provides further evidence for EGCG binding to the same site as DHF. A similar $pK_a$ shift was assigned to Glu$^{30}$ for methotrexate binding to human DHFR (12), which is readily explained by (a) the high degree of sequence homology between the bovine and human enzymes, (b) the published structure of the human enzyme with methotrexate bound (15), and (c) our modeling of EGCG into the active site of human DHFR described below and shown in Fig. 1B.

Molecular Modeling of the Interaction between EGCG and Dihydrofolate Reductase. On searching the available ligand-bound human DHFR structures in the Protein Data Bank (10), we identified a 1.8-Å structure (Protein Data Bank accession code 1S3V; ref. 9) containing a tetrahydroquinazoline antifolate ligand, TQD (Fig. 1), as the best available structural match for EGCG. Using the position of TQD as a guide, EGCG was docked into this protein structure and the EGCG-protein composite was then energy minimized (Fig. 1B). Comparison with a range of other DHFR structures containing folate or various inhibitors showed that most of the EGCG lies within the consensual substrate/inhibitor envelope, with the exception of the nonester trihydroxybenzene moiety. To accommodate this ring, the Leu$^{37}$ side chain is required to adopt a different orientation; a precedent for this movement is provided by the crystal structure of a Tyr$^{37}$ mutant, which displays a similar geometry at this residue (16). There are specific hydrogen bonding interactions, most notably that involving Glu$^{30}$. For folate and methotrexate, adjacent heterocyclic and amino nitrogens of the ligand form a pair of hydrogen bonds with the two oxygens of the Glu$^{30}$ side chain (both O·N distances ≤ 2.8 Å). In contrast, EGCG has only a single phenolic OH group available for hydrogen bonding to Glu$^{30}$ (O·O distance 2.7 Å). This is consistent with the $pK_a$ data discussed above and could explain the soft drug character of this catechin. Other EGCG-protein contacts, shown in Fig. 1B, are similar to those found for TQD.

The difference in the type of inhibition exhibited by EGCG with bovine and chicken DHFR must be due to a difference in primary sequence and hence the three-dimensional structures of these enzymes. The DHFRs from humans, cows, and chickens are quite similar, with sequence identities of 75%, 76%, and 87% for the bovine and chicken DHFR must be due to a difference in primary sequence and hence the three-dimensional structures of these enzymes. The DHFRs from humans, cows, and chickens are quite similar, with sequence identities of 75%, 76%, and 87% for the sequence analysis of our model showed only one residue within 4 Å of the EGCG ligand that is variable in the three sequences, namely, residue 31. This residue is located at the active site of DHFR and is Tyr in chicken, but Phe in human and bovine DHFR. Phe$^{31}$ has the same side chain conformation as Tyr$^{31}$ in some, but not all, of the human structures. Because this side chain has quite extensive van der Waals contacts with the EGCG ligand in our model (see Fig. 1), and interacts with folate and inhibitors such as methotrexate and trimethoprim, its mutation could have a noticeable effect on ligand binding. Indeed, the crystal structure of chicken liver DHFR complexed with NADP$^+$ and biotin showed two alternative conformational changes for Tyr$^{31}$, and the implications of this observation in terms of the catalytic mechanism have been discussed (17).

Inhibition of Dihydrofolate Reductase by EGCG in Cancer Cells. To determine whether EGCG inhibits DHFR activity in vivo, a mouse lymphoma cell line (L1210) was incubated with various concentrations of EGCG in a standard folate medium. EGCG significantly inhibited L1210 growth in a concentration-dependent manner (IC$_{50}$ = 20 μmol/L). If this was specifically due to inhibition of DHFR activity by EGCG, cell growth should be restored in HT...
medium. Antifolates block the de novo biosynthesis of thymine, purines, and pyrimidines by inhibiting the synthesis of 5,6,7,8-tetrahydrofolate, an essential cofactor in these biosynthetic pathways. Cells that express hypoxanthine-guanine phosphoribosyltransferase, an enzyme essential for the recycling of purine nucleotides, can survive in the presence of antifolates in HT medium. Control experiments showed that the inhibition of growth of L1210 cells by methotrexate was greatly attenuated in HT medium (data not shown). Figure 3A shows the time-dependent inhibition of L1210 growth by 20 μmol/L EGCG. Although L1210 grown in HT medium showed a high level of inhibition reversal (Fig. 3A), complete reversal was not obtained after the second day of the experiment. This partial lifting of EGCG inhibition in HT medium is most likely due to secondary effects of EGCG at the concentration used in this assay. EGCG has been reported to have pro-oxidant activity in several cell lines (e.g., hepatoma cells; ref. 18). The production of reactive oxygen species has been associated with the inhibition of cancer cell growth by tea polyphenols (19). The inhibition of L1210 growth by EGCG was partially lifted by the inclusion of the antioxidant ascorbic acid in the reaction medium (Fig. 3A). Similar results were obtained by cotreating the cells with N-acetylcysteine (a glutathione precursor and scavenger of reactive oxygen species) or superoxide dismutase. Growing L1210 in HT medium containing ascorbic acid (Fig. 3A), N-acetylcysteine, or superoxide dismutase completely removed the inhibitory effect of EGCG. These data provide strong evidence that the major site of action of EGCG in vivo is DHFR.

Further evidence for in vivo inhibition of DHFR by EGCG is provided by experiments with lymphoma cells grown in RPMI medium with low folate levels (Fig. 3B and C). These experiments were designed to investigate whether folate depletion has an effect on the sensitivity to EGCG. Cancer cell lines in standard cell-culture medium are exposed to relatively high folate levels compared with the folate levels in human plasma (19). Consequently, the concentration of folates in the culture medium could affect the extent of EGCG inhibition of cell growth. The concentrations of EGCG needed to inhibit L1210 growth in media with low folate levels were much lower than were needed in a standard folate medium (Fig. 3B). In a low-folate medium supplemented with 30 nmol/L folic acid the IC50 value decreased to 3 μmol/L, and it was then possible to study the time-dependent inhibition of L1210 growth at a lower concentration of EGCG (1 μmol/L). Under these more physiologically relevant growth conditions, inhibition by EGCG was completely reversed in HT medium. These data show that inhibition of DHFR activity could be the major mechanism of the antitumor action of EGCG at physiologic concentrations of folate substrates and blood serum levels of EGCG.

We have shown for the first time that gallated tea polyphenols act as DHFR inhibitors in vitro and in vivo, at concentrations usually found in the blood of tea drinkers. The “soft” character of such compounds could be developed for use in the prevention and treatment of cancer with significantly reduced side effects compared with those of the DHFR inhibitors currently in use in chemotherapy, such as methotrexate. An advantage of EGCG is its differential effects on normal and cancer cells. Importantly, at physiologically attainable concentrations, EGCG kills cancer cells through apoptosis, but has little or no effect on normal cells. Inhibition of DHFR by EGCG explains this differential effect because antifolate compounds are more active on cancer cells, which generally have a higher turnover of DNA. Induction of apoptosis can provide highly effective chemotherapeutic and chemopreventative strategies for cancer control. Many chemopreventative agents act through the induction of apoptosis as a mechanism for the suppression of carcinogenesis by eliminating genetically damaged cells, initiated cells, or cells that have progressed to malignancy. Thus, the soft character of EGCG together with its ability to induce apoptosis through DHFR inhibition provides a convincing explanation for the epidemiologic data on the prophylactic effects of diets high in gallated polyphenols for certain forms of cancer.

We conclude that gallated polyphenols and their derivatives have considerable potential for clinical application as anticarcinogenic agents and as antibiotics and for the treatment of psoriasis (1–3). Our data may also explain why neural tube defects such as anencephaly and spina bifida, which are usually
associated with folic acid deficiency, have been linked to high levels of maternal green tea consumption during the periconceptional period (20).

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