Synthesis and Biological Activity of a 3,4,5-Trimethoxybenzoyl Ester Analogue of Epicatechin-3-Gallate

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RUNNING TITLE: *Synthesis and Activity of a Tea Catechin Analogue*

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[a]Abbreviations: EGCG, (-)-epigallocatechin-3-gallate; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EC, (-)-epicatechin; DNMT, 5-cytosine DNA methyltransferase; DHFR, dihydrofolate reductase; DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; TS, thymidylate synthase; MTX, methotrexate; TMP, trimethoprim; TMECG, 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin; DMAP, 4-(dimethylamino)pyridine; Trolox, 6-hydroxy-2.5,7,8-tetramethylchroman-2-carboxylic acid; TEAC, Trolox equivalent antioxidant activity; SOD, superoxide dismutase; rHDHFR, recombinant human DHFR; TQD, (R)-6-{[methyl-(3,4,5-trimethoxyphenyl)-amino]methyl}-5,6,7,8-tetrahydroquinazoline-2,4-diamine; MTHFR, methyltetrahydrofolate reductase; ATCC, American type tissue culture collection; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HeM, human epidermal melanocytes; HRP, horseradish peroxidase; ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).

Summary

Despite presenting bioavailability problems, tea catechins have emerged as promising chemopreventive agents due to their observed efficacy in various animal models. To improve the stability and cellular absorption of tea polyphenols, we developed a new catechin-derived compound, 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG), which has shown significant antiproliferative activity against several cancer cell lines, especially melanoma. The presence of methoxy groups in its ester-bond gallyl moiety drastically decreased its antioxidant and prooxidant properties without affecting its cell-antiproliferative effects, and the data indicated that the 3-gallyl moiety was essential for its biological activity. As regards its action mechanism, we demonstrated that TMECG binds efficiently to human dihydrofolate reductase and downregulates folate cycle gene expression in melanoma cells. Disruption of the folate cycle by TMECG is a plausible explanation for its observed biological effects and suggests that, like other antifolate compounds, TMECG could be of clinical value in cancer therapy.

KEYWORDS: *Tea catechins; antioxidants; dihydrofolate reductase; melanoma; folate cycle.*

Introduction

Green tea, one of the most popular and commonly consumed beverages in the world, is an important source of flavonoids called catechins (Chart 1). Among them, (-)-epigallocatechin-3-gallate (EGCG[a]) is the most abundant (40-60%), followed by (-)-epicatechin-3-gallate (ECG; 10-20%), (-)-epigallocatechin (EGC; 10-20%) and (-)-epicatechin (EC; 4-6%); in sum, they contribute to more than 200 mg of catechin per cup of green tea. Several *in vivo* experiments have shown the chemopreventive effects of EGCG against all stages of carcinogenesis (initiation, promotion, and progression) in animal models of breast, lung, skin, prostate and colon cancers.[1,2] There has been extensive investigation into the ways by which EGCG might act in cancer prevention, and recent attempts to define the molecular action mechanism of green tea components have found that EGCG affects several molecular targets and pathways of carcinogenesis.[2,3] The observed prooxidant nature of EGCG appears to conflict with the generally recognized concept that tea polyphenolics act as antioxidants.[4] Several studies have shown that the trihydroxyphenyl B-ring is the principal site for prooxidant reactions, regardless of the presence of the 3-gallyl moiety.[5,6] However, the presence of hydroxyl groups in the 3-gallyl moiety may enhance the antioxidant potential of tea catechins.[5] The 3-gallyl moiety of catechins has also seen to be essential for the inhibition of several of their proposed enzymatic targets, such as the proteasome,[7] 5-cytosine DNA methyltransferase (DNMT)[8] and dihydrofolate reductase (DHFR).[9-11]

Based on the observation that some tea polyphenols possess similar chemical structures to classical and non-classical antifolates (Chart 2), we hypothesized that tea catechins could inhibit DHFR activity. Recently, we reported that ester bound gallate catechins isolated from green tea are potent inhibitors of DHFR activity at concentrations found in

the serum and tissues of green tea drinkers (0.1–1.0 μM).[9-11] DHFR (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) in the presence of coenzyme NADPH as follows: DHF + NADPH + $H^+ \rightarrow$ THF + $NADP^+$. This enzyme is necessary for maintaining intracellular pools of THF and its derivatives, which are essential cofactors in one-carbon metabolism. Coupled with thymidylate synthase (TS),[12] it is directly involved in thymidylate (dTMP) production through a *de novo* pathway. DHFR is therefore pivotal in providing purines and pyrimidine precursors for the biosynthesis of DNA, RNA and amino acids. In addition, DHFR is the target enzyme for antifolate drugs, such as the antineoplastic drug methotrexate (MTX) and the antibacterial drug trimethoprim (TMP).[13] The antifolate character of EGCG and ECG could explain their anticarcinogenic and chemopreventive action, their anti-inflammatory properties and even their claimed ability to promote weight loss.[11] Moreover, this mechanism may also explain the high antimicrobial and antimycotic action of these compounds, which protect against dental caries and infections produced by pathogens such as *Helicobacter pylori* or *Candida albicans*.[14-17]

However, despite these excellent properties, tea catechins have at least one limitation, their low bioavailability.[18] Factors influencing this low bioavailability could be related with their low stability in neutral or slightly alkaline solutions and their inability to cross cellular membranes.[19] In an attempt to solve such bioavailability problems, we synthesized a 3,4,5-trimethoxybenzoyl analogue of ECG [3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin; TMECG] (Chart 3), which showed significant antiproliferative activity against different cancer cell lines. Although TMECG has been synthesized previously,[20] its biological properties have scarcely been studied and its molecular structure has not been

characterized. The purpose of the present study was to present an inexpensive alternative for TMECG synthesis, accompanied by a high degree of recovery, and to study the biological activity of this compound in different cancer cell systems. In addition, any change in the antioxidant/prooxidant profile of TMECG with respect to naturally occurring tea catechins, but where the 3-gallyl moiety is maintained, might contribute to understanding the mechanism/s of action of this biologically active compound.

Results and Discussion

Syntheses. Although TMECG was previously synthesized,[20] experiments performed in our laboratory to synthesize this ECG analogue following the described methodology rendered very low recovery. A major problem was associated with unspecific blockage of the 3-hydroxyl group of the epicatechin after the benzylation reaction with benzyl bromide and K_2CO_3. It is well known that the benzylation of epicatechin provides a product of low purity even after recrystallization.[21] Thus, TMECG was successfully synthesized following the five-step reaction sequence shown in Scheme 1, starting from the commercially available catechin, which is considerably cheaper than epicatechin. In the first step, the phenolic hydroxyl groups, but not of the alcoholic hydroxyl group, were protected by treating the catechin with benzyl bromide in the presence of K_2CO_3, thus yielding the corresponding tetra-*O*-benzylcatechin **1** in a 45% yield.[22] Next, the tetra-*O*-benzylepicatechin **3** was obtained from **1** following a synthetic methodology which involved an inversion of the stereochemistry at C-3 through an oxidation-reduction process. Oxidation of **1**, using 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one (Dess-Martin periodinane)[23] in moist CH_2Cl_2, gave the ketone derivative **2**, which was subsequently reduced to **3** with lithium tri-*sec*-butylborohydride (L-Selectride) in THF at -

78 ºC. At this point, 3,4,5-trimethoxybenzoyl chloride, previously prepared by reaction of the corresponding carboxylic acid with PCl_5 in toluene under reflux temperature, was esterified with **3,** in a CH_2Cl_2 solution and in the presence of 4-(dimethylamino)pyridine (DMAP), giving **5** in a 71% yield. In the final step of this synthesis, the benzyl-protecting groups were removed, giving rise to **6** (62% yield), using a strategy based on a hydrogenolysis reaction which was carried out by treating **5** over 20% $Pd(OH)_2/C$ in THF/MeOH. Compounds **5** and **6** were characterized by ^{1}H and ^{13}C-NMR, elemental analysis and MSFAB spectrometry.

Stability and Cellular Uptake of TMECG. The biological effects of tea catechins have been extensively investigated in cell lines, but the stability and cellular uptake of these compounds are poorly understood. The most active tea polyphenols are unstable in neutral or alkaline medium. Zhu and co-workers[24] demonstrated that ester cleavage of gallate catechins is unlikely and, therefore, oxidation at different molecular levels (especially in the phenolic hydroxyl groups of rings B and D) is the most plausible mechanism for explaining their instability. Thus, at high pH values, the basic environment can easily deprotonate the phenolic groups, generating phenoxide anions. The anion form is much more reactive toward electrophilic agents, such as free radicals, and also forms the semiquinone radical, which can undergo further dimerization or other reactions. As the pH value of the intestine and body fluid is neutral or slightly alkaline, these tea catechins will be very unstable inside the human body, leading to reduced bioavailability.[24,25] The stability of EGCG has been studied in detail and seen to vary with the cell culture conditions.[19] EGCG is not stable in cell culture systems, and its oxidation leads to the formation of dimers and H_2O_2. Under many conditions, the half-life of EGCG is <2 h in the presence of HT-29 human colon adenocarcinoma cells and even shorter in the absence

of cells.[19] Methoxy protection of the reactive hydroxyls groups in the D-ring of ECG yielded a stable derivative in cell culture conditions. TMECG was considerably more stable than ECG in a culture medium in the absence of cells. Although the first time point was not statistically different for either compound, the successive time points showed statistically significant differences. The data showed that more than 75% of the initial concentration of TMECG remained in the culture medium after an incubation period of 2 days, while only 30% of the initial concentration of ECG remained at this time (Figure 1A). Another factor reducing the bioavailability of tea catechins is the low capacity of these compounds to cross cell membranes. To study the cellular uptake of TMECG, we used Caco-2 cell monolayers, which acted as a model of the human intestinal epithelium.[26,27] Figure 1B shows the concentration of ECG and TMECG in culture media in the presence of Caco-2 cells. As can be seen, the TMECG concentration rapidly decreased in the cell medium as a consequence of cellular uptake with a maximal decrease after 4 hours' exposure. The more hydrophilic character of ECG made its cellular uptake slower (Figure 1B). The data indicated that TMECG is more efficiently transported through the Caco-2 cell membranes than its natural counterpart tea catechin.

Biological Activity of TMECG on Cancer Cells. Tea polyphenols have demonstrated their antiproliferative activity against human cells lines from breast,[28] lung,[29] colon,[30] and melanoma,[31] among others. To observe the possible wide spectrum of the antiproliferative activity of TMECG we investigated its effects against different human cancer lines obtained from breast (MCF7), lung (H1264), colon (Caco-2) and melanoma (SkMel-28) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effectiveness of TMEG on these cells followed the order SkMel-28 > H1264 > Caco-2 > MCF7, with a half maximal inhibitory concentration (IC_{50}) at 6 days of

2.9 ± 0.5, 18 ± 2.0, 33 ± 3.1 and 80 ± 5.5 μM, respectively. The calculated IC_{50}-values were statistically different between cell lines but in the same concentration range to those reported for the activity of TMECG on other cellular systems.[20] The factors that modulate this different cell response to TMECG are currently being investigated in our laboratory. Next, we investigated the cancer cell inhibitory capacity of TMECG in comparison with naturally occurring tea polyphenols. After 6-days' exposure of Caco-2 cells to 25 μM catechins, EGCG, ECG and TMECG showed remarkable growth inhibition activity compared with an untreated control (Figure 2A). However, catechins lacking the 3-gallyl moiety, such as EGC and EC, showed much lower growth inhibitory activity (Figure 2A). Smkel-28 melanoma cells were highly susceptible to TMECG treatment and, after 6-days' exposure to 20 μM TMECG, the cells showed microscopic signs of apoptosis, including cell shrinkage, loss of cell-cell contact and the fragmentation of plasmatic and nuclear membranes (Figure 2B).

Antioxidant versus Prooxidant Activity of TMECG. Although claims concerning the beneficial health effects of green tea consumption are based on many epidemiological and laboratory studies, the exact action mechanism of its polyphenolic compounds is subject to continuous debate. Many hypotheses and molecular targets for EGCG and other tea catechins have been proposed in the last ten years. Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action.[32] This is generally considered to reflect their ability to scavenge endogenously generated oxygen radicals or those radicals formed by various xenobiotics, radiation, etc. However, some data in the literature suggest that the antioxidant properties of polyphenolic compounds may not fully account for their chemopreventive effects.[33] Most plant polyphenols possess both antioxidant and prooxidant properties[34] and it has

been frequently suggested that the prooxidant action of polyphenolics may be an important mechanism in their antiproliferative and apoptosis-inducing properties.[35] Whatever the case, the mechanism whereby tea catechins change from antioxidant to prooxidant activity, and vice versa, is uncertain. To test whether the biological activity of TMECG was related with its antioxidant and/or prooxidant properties, we determined the antioxidant and prooxidant properties of this compound and compared it with the properties of other natural occurring catechins in green tea.The antioxidative activity of catechins was evaluated by measuring the Trolox equivalent antioxidant capacity values (TEAC).[36] This method measures the relative ability of antioxidant substances to scavenge the radical cation 2,2'-azino-bis-(3-ethylbenzthia-zoline-6-sulfonic acid) ($ABTS^{\cdot+}$) compared with a standard amount of the synthetic antioxidant Trolox (6-hydroxy-2.5,7,8-tetramethylchroman-2-carboxylic acid), an aqueous soluble vitamin E analogue. The TEAC value is defined as the concentration of standard Trolox with the same antioxidant capacity as 1 mM concentration of the antioxidant compound under investigation. The results showed that the compounds with the most hydroxyl groups apparently exert the greatest antioxidant activity (Figure 3A), with ECG and EGCG at 4.9 and 4.8 mM, respectively, being more active antioxidants than vitamin E (TEAC = 1.0 mM). The enhanced values for the catechin-gallate esters compared with non-gallated catechins (EC or EGC) reflect the additional contribution from the trihydroxybenzoate, gallic acid, in ring D. Substitution of the hydroxyl groups in ring D by methoxy groups decreased the antioxidant activity of TMECG (TEAC = 1.9 ± 0.1 mM) with respect to the other catechin-gallate esters but had no appreciable effect on the growth inhibition of Caco-2 cells (Figure 2A). It therefore seems that the antioxidant potential of this synthetic catechin was not responsible for the described biological effects.

In addition, phenolics can exert prooxidant effects by autoxidation, in which the initial step leads to semiquinone and superoxide radical formation.[37] These radicals are very reactive and may damage major macromolecules such as proteins or DNA. Moreover, both radicals will accelerate autoxidation, generating H_2O_2 in the process. It has been suggested that the prooxidant activity of catechins may be due to the flavonoid unit of catechin (ring B). An additional group in this unit, as occurs in EGC and EGCG, enhances the prooxidant activity of the catechins.[32] Figure 3B, which shows the extent of NADPH oxidation in the presence of several catechins, seems to confirm this hypothesis. EGC and EGCG enhanced NADPH oxidation which was inhibited by the presence of superoxide dismutase (SOD). However, the oxidation of NADPH in the presence of ECG, EC or TMECG was negligible throughout the experiments. If the biological activity of catechins is related with their prooxidant activity we would expect a stronger activity in the presence of either EGC or EGCG; however, EGC was inactive and EGCG showed similar activity to ECG and TMECG (Figure 2A). As observed in Figure 2A, the effect of catechins on Caco-2 cells was more related with the presence of a gallate ester moiety, leading us to check other hypotheses to explain the biological activity of TMECG.

Binding of TMECG to Human DHFR. Green tea extracts containing significant amounts of tea catechins strongly inhibited the activity of recombinant human DHFR (rHDHFR).[11] In order to detect which components of these extracts were responsible for such inhibition we assayed DHFR activity in the presence of EC, EGC, ECG and EGCG. The results showed that both ECG and EGCG were potent inhibitors of the human enzyme, while polyphenols lacking the ester bound gallate moiety (EGC and EC) did not inhibit rHDHFR (Figure 4A). These results indicate that the ester-bound gallate moiety is essential for the inhibition of this enzyme, as has been determined in the case of bovine

DHFR.[9,10] TMECG with an ester-bound gallate unit was also a good inhibitor of rHDHFR (Figure 4A). The effective binding of TMECG to free rHDHFR was determined by following the decrease in enzyme fluorescence that occurs on formation of the enzyme-inhibitor complex (Figure 4B). When rHDHFR fluorescence was excited at 290 nm its emission spectrum showed a maximum at 340-350 nm. The binding of TMECG quenched this fluorescence and the data showed that the dissociation constant of free rHDHFR for TMECG (2.1 ± 0.2 μM) was in the same order as that for EGCG (0.92 μM) and ECG (1.78 μM). As expected, the addition of EGC to rHDHFR did not modify enzyme fluorescence (Figure 4B). Structural modeling of TMECG into human DHFR indicated that the compound is orientated in a similar way to that described for EGCG,[9] with specific hydrogen bonding interactions, most notably involving Glu-30 (O···O distance 2.6 Å) (Figure 4C). Comparison with a range of other DHFR structures containing folate or various inhibitors showed that most of the TMECG lies within the consensual substrate/inhibitor envelope, with the exception of the non-ester trihydroxybenzoyl moiety (ring B). The ester-bound gallate moiety (ring D) is accommodated in an amphiphilic region of DHFR involving residues Ile7, Gln35, Asn64 and Leu67.[38] Although folate, TQD, MTX and tea catechins differ significantly in terms of their structural formulae, they have a similar 3D shape,[9] which appears to be an important determinant in their binding to DHFR.

TMECG Modulates Folic Acid Cycle Gene Expression. The folate cycle plays a central role in cell metabolism (Scheme 2). Among its important functions are the delivery of one-carbon units to the methionine cycle for use in methylation reactions, and the synthesis of pyrimidines and purines. Several enzymes, including DHFR, TS and methyltetrahydrofolate reductase (MTHFR), participate in the activation and regeneration

of folic acid coenzymes. Several studies have shown that the protein and mRNA levels of TS and DHFR are higher in tumor tissues and cancer cells than in their normal counterpart.[39,40] Tumors with high levels of these enzymes are thought to undergo more active cellular proliferation, which, in turn, is associated with tumor invasiveness and metastasis. To investigate whether TMECG could interfere with folate metabolism in cancer cells, the levels of expression of these folate cycle genes were analyzed in melanoma SkMel-28 cells using real time PCR. As observed in other tumor tissues and cells, the genes involved in the metabolism of folic acid were highly overexpressed in melanoma cells compared with normal melanocytes (Figure 5). The levels of DHFR, TS and MTHFR mRNAs were calculated to be about 400-, 22- and 4-fold higher, respectively, in melanoma cells than in normal melanocytes. Treatment of SkMel-28 with TMECG produced a substantial and rapid downregulation of these genes, involving significant changes in the mRNA levels of the genes at 24 h of treatment. DHFR, TS and MTHFR mRNA levels were similar to that detected in normal melanocyte cells after 5 days of TMECG treatment (Figure 5). The data indicated that TMECG disturbs the folate metabolism in melanoma cells and suggest that this might be the mechanism by which TMECG induces cell growth inhibition and death.

Conclusions. The synthesis of a 3,4,5-trimethoxybenzoyl ester analogue of ECG was successfully carried out by using catechin and inverting the stereochemistry at carbon-3. TMECG was more stable and more effectively transported in colon cancer cell than its natural counterpart ECG. In evaluating its antiproliferative activity, we showed that TMECG efficiently inhibited the growth of different human cells including cancer lines from breast, lung, colon and skin. The presence of methoxy groups in its ester-bond gallyl moiety decreases its antioxidant and prooxidant properties without affecting its cell-

antiprofiferative effects. The data indicated that the 3-gallyl moiety is essential for the biological activity of TMECG. Recent investigations into the action mechanism of tea catechins reveal that their 3-gallyl moiety is required for the binding and inhibition of several enzymatic targets including the proteasome,[7] DNMT[8] or DHFR.[9-11] This moiety was also proposed as being necessary to inhibit folic acid uptake in Caco-2 cells.[28] We have shown here that TMECG effectively binds to human DHFR and controls the expression of the genes involved in folate metabolism. We therefore conclude that TMECG, by inhibiting DHFR and/or inhibiting folate transport and/or acting on another proposed cellular target, acts as an antifolate metabolite, whose action would result in disruption of the cell folate cycle (Scheme 2). Folate cycle disruption has an important effect on cells because the depletion of folate coenzymes inhibits DNA synthesis, decreases the cellular production of nucleotides, alters DNA methylation patterns and affects polyamine synthesis (Scheme 2). These effects are more drastic in rapidly proliferating cancer cells since they have a high turnover of DNA and, therefore, higher folate requirements. In conclusion, TMECG could be an effective anticancer drug which shares an action mechanism with other ester gallate catechins. Moreover, the presence of a more lipophilic 3-gallyl moiety that enhances the stability and cellular uptake could mitigate the main weakness of tea catechins - their poor bioavailability.

Experimental Section

All reactions were carried out using solvents which were dried by routine procedures. All melting points were determined on a Kofler hotplate melting-point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 MHz. The following abbreviations are used to represent the multiplicity of the signals: s (singlet), d

(doublet), dd (double doublet), m (multiplet), and q (quaternary carbon atom). Chemical shifts refer to signals of tetramethylsilane in the case of ^{1}H and ^{13}C NMR spectra. The FAB^{+} mass spectra were recorded on a Fisons AUTOSPEC 5000 VG spectrometer, using 3-nitrobenzyl alcohol as a matriz.

Compounds **1**, **2** and **3** were obtained using the experimental procedures described elsewhere.[21]

3,4,5-Trimethoxybenzoyl chloride, 4: A mixture of 3,4,5-trimethoxybenzoic acid (3.0 g, 14.14 mmol) and phosphorous pentachloride (3.53 g, 16.95 mmol) in dry toluene (50 ml) was stirred at reflux temperature and under nitrogen for 3 h. The reaction was allowed to reach room temperature and the solvent was removed under vacuum. The product was sufficiently pure to be used in the following step without further purification.

5, 7, 3´, 4´-Tetra-*O*-benzyl-3-(3´´, 4´´, 5´´-trimethoxybenzoyl)-(-)-epicatechin, 5: To a solution of **3** (0.547 g, 0.84 mmol) and DMAP (0.257 g, 2.10 mmol) in dry CH_2Cl_2 (50 ml) a solution of 3, 4, 5-trimethoxybenzoyl chloride **4** (0.386 g, 1.68 mmol) in the same solvent was added dropwise under nitrogen. The reaction mixture was stirred at room temperature for 24 hours and then a solution of saturated sodium bicarbonate (30 ml) and ethyl acetate (50 ml) was added. Afterwards, the mixture was extracted twice with water (2x100 ml). The organic layers were dried with anhydrous magnesium sulfate and the solvent removed under vacuum. The resulting red oil was chromatographed on a silica gel column using *n*-Hex/AcOEt (6:4, v:v) as solvent (R_f = 0.64). The solvent was removed under reduced pressure and the solid was recrystallized from Et_2O/*n*-Hex to obtain a white solid (yield = 71%). MS (FAB^{+}) m/z (%) 845 (M^{+}+1, 10). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30-7.43 (m, 20H, Ph), 7.17 (s, 2H, H2´´ and H6´´), 7.13 (d, 1H, ^{4}J = 1.9 Hz, H2´), 7.07 (dd, 1H, ^{3}J = 8.4 Hz, ^{4}J = 1.9 Hz, H6´), 6.89 (d, 1H, ^{3}J = 8.4 Hz, H5´), 6.30 (d, 1H, ^{4}J = 2.2

Hz, H6), 6.28 (d, 1H, ^4J = 2.2 Hz, H8), 5.11 (s, 4H, 2xCH_2O), 5.03 (s, 2H, CH_2O), 5.02 (s, 2H, CH_2O), 4.97 (d, 1H, ^3J = 11.7 Hz, H2), 4.87 (d, 1H, ^3J = 11.7 Hz, H3), 3.83 (s, 3H, OCH_3), 3.80 (s, 6H, OCH_3), 3.10 (m, 2H, H4). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.9 (q, -COO), 158.4 (q, Ar-O), 157.6 (q, Ar-O), 155.2 (q, Ar-O), 152.5 (2xq, Ar-O), 148.6 (q, Ar-O), 142.0 (q, Ar-O), 136.8 (q, $PhCH_2$), 136.7 (q, $PhCH_2$), 136.5 (q, $PhCH_2$), 136.4 (q, $PhCH_2$), 130.8 (q, C1´), 128.3 (CH, $PhCH_2$), 128.2 (CH, $PhCH_2$), 128.1(CH, $PhCH_2$), 128.0 (CH, $PhCH_2$), 127.6 (CH, $PhCH_2$), 127.5 (CH, $PhCH_2$), 127.4 (2xCH, $PhCH_2$), 127.1 (CH, $PhCH_2$), 127.0 (CH, $PhCH_2$), 126.9 (CH, $PhCH_2$), 126.8 (CH, $PhCH_2$), 124.7 (q, C1´´), 119.6 (CH, C6´), 114.4 (CH, C6), 113.6 (CH, C2´), 106.7 (CH, C2´´ y C6´´), 100.5 (q, C4a), 94.2 (CH, C6), 93.5 (CH, C8), 71.2 (CH_2, CH_2Ph), 70.9 (CH_2, CH_2Ph), 69.8 (CH_2, CH_2Ph), 69.6 (CH_2, CH_2Ph), 68.5 (CH, C3), 60.5 (CH_3, OCH_3), 55.8 (CH_3, OCH_3), 25.5 (CH_2, C4). Anal. ($C_{53}H_{48}O_{10}$) C, H: calcd, 75.34, 5.73; found, 75.57, 5.94.

3-*O*-(3, 4, 5-Trimethoxybenzoyl)-(-)-epicatechin, 6: Under normal pressure, a solution of **5** (0.600 g, 0.71 mmol) and 10% Pd/C (0.06 g of palladium, 0.56 mmol) in THF/MeOH (1:1,40 ml) was treated with molecular hydrogen. The solution was stirred for 18 h at room temperature and then filtered on a Celite pad, which was then washed with methanol (300 ml). The solvent was removed under vacuum and the resulting solid was recrystallized from Et_2O (yield = 62 %). MS (FAB^+) m/z (%) 485 (M^++1, 28). ^1H NMR (Acetone-d_6, 400 MHz) δ 8.31 (bs, 1H, OH), 8.10 (bs, 1H, OH), 7.94 (bs, 1H, OH), 7.80 (bs, 1H, OH), 7.14 (s, 2H, H2´´ y H6´´), 7.11 (d, 1H, ^4J = 2.0 Hz, H2´), 6.90 (dd, 1H, ^3J = 8.2 Hz, ^4J = 2 Hz, H6´), 6.79 (d, 1H, ^3J = 8.2 Hz, H5´), 6.03 (d, 1H, ^4J = 2.2 Hz, H6), 6.02 (d, 1H, ^4J = 2.2 Hz, H8), 5.48 (m, 1H, H3), 5.19 (bs, 1H, H2), 3.80 (s, 6H, OCH_3), 3.73 (s, 3H, OCH_3), 3.10 (m, 1H, Hgem, H4), 3.05 (m, 1H, Hgem, H4). ^{13}C NMR (Acetone-d^6, 100 MHz) δ 165.9 (q, -COO), 157.9 (q, Ar-O), 157.4 (q, Ar-O), 156.8 (q, Ar-O), 154.0 (q, Ar-

O), 145.7 (q, Ar-O), 145.4 (q, Ar-O), 143.3 (q, Ar-O), 131.4 (q, C1´), 126.2 (q, C1´´), 118.7 (CH, C6´), 115.6 (CH, C5´), 114.6 (CH, C2´), 107.6 (CH, C2´´ y C6´´), 98.5 (q, C4a), 96.4 (CH, C7), 95.5 (CH, C9), 77.7 (CH, C2), 70.7 (CH, C3), 60.6 (CH_3, CH_3O), 56.4 (CH_3, CH_3O), 26.0 (CH_2, C4). Anal. ($C_{25}H_{24}O_{10}$) C, H: calcd, 61.98, 4.99; found, 61.69, 5.24.

Materials. Highly purified tea polyphenols, EGCG (>95%), ECG (>98%), EGC (>98%) EC (>98%), and (-)-catechin (>98%) were purchased from Sigma Chemical Co. (Madrid, Spain). rHDHFR was purchased from Sigma and dialyzed exhaustively against distilled water prior to use. The enzyme concentration was determined by MTX titration of enzyme fluorescence.[41] DHF (90%) was obtained from Aldrich Chemical Co. (Madrid, Spain) and NADPH from Sigma. The concentrations of NADPH and DHF were determined enzymically at 340 nm using DHFR and a molar absorbance change ($\Delta\varepsilon$) for the reaction of 11800 $M^{-1}cm^{-1}$ at this wavelength.[42] SOD from bovine liver was obtained from Sigma and used without further purification.

Cell Cultures. Human cancer cells lines (SkMel-28, MCF7, H1264 and Caco-2) were obtained from the American Type Tissue Culture Collection (ATCC) and were maintained in appropriate culture media supplemented with 10% fetal calf serum (FCS) and antibiotics, under standard tissue culture conditions. Cell injury was evaluated by a colorimetric assay for mitochondrial function using the MTT cell proliferation assay.[43] The assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and to form dark blue formazan crystals, which cannot pass through cell membranes, thus so they accumulated within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created and the color can then be quantified at 570 nm. For this assay,

cells were plated in a 96-well plate at a density of 1000 cells/well and grown until they reached 50-60% confluence. Human epidermal melanocytes (HeM) were supplied by Gentaur (Brussels, Belgium) and were cultured in HAM-F10 medium supplemented with 10% FCS, antibiotics and the human melanocyte growth supplement (Gentaur).

Stability and Cellular Uptake of Catechins. Stability and cellular uptake were determined by the difference between the initial concentration of catechin and that calculated at specific times in the cellular medium, and expressed as a percentage of catechin at zero time. Catechin concentrations were determined by HPLC analysis. Briefly, 50 μL of cellular medium were injected into a HPLC system (Hitachi, LaChrom Elite) equipped with a 250 x 4.6-mm C18 column and a UV-Vis detector. The mobile phase was 0.1 M sodium dihydrogen phosphate buffer (pH 2.5) containing 0.1 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)/acetonitrile (87/13 v/v), and the flow rate was 1 mL/min. The column was maintained at 30 °C. Catechins were identified by their characteristic elution times and their concentrations were calculated with respect to calibration curves for known concentrations of catechins.

Antioxidant Activity. The antioxidant capacity was measured using a method based on the abilities of different substances to scavenge the $ABTS^{\cdot+}$ radical cation compared with a standard antioxidant (Trolox) in a dose-response curve.[36] $ABTS^{\cdot+}$ radical cation was prepared by oxidation of ABTS (150 μM) in the presence of H_2O_2 (75 μM) and horseradish peroxidase (HRP) (0.25 μM) in citrate-phosphate buffer, pH 4.5. This solution was then diluted in 5 mM phosphate buffered saline (PBS) pH 7.4 to an absorbance of 0.70 (± 0.02) at 734 nm and pre-incubated at 30 ºC prior to use. Fresh $ABTS^{\cdot+}$ radical cation solution was prepared each day. 2.5 mM Trolox was prepared in PBS for use as stock

standard. Fresh working standards were prepared daily by diluting 2.5 mM Trolox with PBS. All catechins were dissolved in PBS to a concentration of 50 μM. After addition of 1 mL of $ABTS^{\cdot+}$ solution to aliquots of Trolox or catechins (1-100 μL, depending on the activity of the particular compound) the solutions were vortexed for exactly 30 s and the absorbance at 734 nm was taken exactly 1 min after initiation of mixing in a Perkin-Elmer Lambda-35 spectrophotometer. PBS blanks were run in each assay. The dose-response curve for Trolox consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation and was based on triplicate determinations. The activities of catechins were assayed at four different concentrations previously determined to be within the range of the dose-response curve. All the measurements were repeated on triplicate samples at these four concentrations. By reference to the Trolox dose-response curve, the mean Trolox equivalent antioxidant capacity (TEAC) value was derived for each catechin compound.

NADPH Oxidation by Catechins. NADPH oxidation in the presence of catechins was determined by following the decrease in absorbance of NADPH at 340 nm in a Perkin-Elmer Lambda-35 spectrophotometer.

DHFR Assays. The activity of DHFR in the absence or presence of catechins was determined at 25 ºC by following the decrease in the absorbance of NADPH and DHF at 340 nm. Experiments were performed in a buffer containing 2-(*N*-morpholino)-ethanesulfonic acid (Mes, 0.025 M), sodium acetate (0.025 M), tris(hydroxymethyl)-aminomethane (Tris 0.05 M), and NaCl (0.1 M) at pH 7.4. To prevent the oxidation of catechins, the reaction mixture contained 1 mM ascorbic acid. Assays were started by adding enzyme. In the absence of the enzyme, the rate of absorbance change was negligible.

Fluorescence Studies. The fluorescence of DHFR is reduced upon binding of substrates and inhibitors, a property that can be used as a convenient method for determining both the enzyme concentration and the dissociation constants of enzyme-ligand complexes. The dissociation constant for the binding of TMECG to free rHDHFR was determined by fluorescence titration in an automatic-scanning Perkin-Elmer LS50B spectrofluorimeter with 1.0 cm light path cells and equipped with a 150 W xenon (XBO) light source. The formation of a binary complex between the enzyme and the ligand was followed by measuring the quenching of the tryptophan fluorescence of the enzyme upon addition of microliter volumes of a concentrated stock solution of ligand. Fluorescence emission spectra were recorded when rHDHFR fluorescence was excited at 290 nm. Titrations were performed in the same buffers as described for DHFR assays. Temperature was controlled at 25 ºC using a Haake D1G circulating bath with a heater/cooler.

Molecular Modeling. Molecular modeling was carried out using the Discover module of Insight II (Insight II, release 2000.1, Accelrys Ltd. Cambridge, UK). Human DHFR X-ray crystal structure 1S3V[44] was retrieved from the protein data bank,[45] and its TQD ligand was used as a template for positioning the TMECG ligand. The composite protein/TMECG model was geometrically optimized within Insight II using the consistent valence force field and steepest descent algorithm to a derivative of 1.0. The refined model was validated within InsightII using Prostat.

RNA Isolation. SkMel-28 and HeM polyA$^+$ mRNA was extracted from 5×10^6 cells using the Illustra Quick Prep Micro mRNA purification kit (GE Healthcare, Barcelona, Spain). mRNA (200 ng) was used to synthesize cDNA using SuperScript First-Strand Synthesis System (Invitrogen, Barcelona, Spain).

Quantitative Real-Time PCR Analysis. cDNA samples (1 μL) were used for real-time PCR in a total volume of 20 μL using SYBR Green Reagent (Applied Biosystems) and specific primers on a 7500 Real Time PCR System of Applied Biosystems. The PCR amplification cycles included denaturation at 95 ºC for 15 min (to activate HotStar Taq DNA polymerase and to minimize primer-dimer contribution) and amplification [over 40-50 cycles including denaturation (94 ºC; 30 s), annealing (55 ºC; 30s), and extension (72 ºC; 1 min)]. All PCR reactions were performed in triplicate and in at least two independent experiments. Non-RT control and negative control samples (without template) were processed in the same manner. The specificity of the amplification was verified by melting curve analysis for all samples, and occasionally by agarose gel electrophoresis. Amplification of target gene sequences were compared against serial dilutions of known quantities of their purified cDNA fragments, and normalized to the abundance of the house-keeping gene β-actin.

Primers for PCR. Primers were designed using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) and synthesized by Invitrogen. The sequence of the primers was as follows: DHFR (forward: 5'-ATGCCTTAAAACTTACTGAACAACCA-3'; reverse: 5'-TGGGTGATTCATGGCTTCCT-3'); TS (forward: 5'-AGATCCAACACA-TCCTCCGCT-3'; reverse: 5'-CCAGAACACACGTTTGGTTGTCAG-3'); MTHFR (forward: 5'-CTTTGGGGAGCTGAAGGACTACTAC-3'; reverse: 5'-CACTTTGTGAC-CATTCCGGTTTG-3').

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Supporting Information Available: ^{1}H and ^{13}C-NMR spectra for compounds **5** and **6** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Legend to Schemes

Scheme 1. Synthesis of 3-*O*-(3,4,5-trimethoxybenzoyl)-(-)epicatechin **6**[a]

Scheme 2. General effects of antifolates on the folic acid cycle. Abbreviations not included in the text: MS, Methionine synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Legend to Figures

Figure 1. (A) Stability of ECG (●) and TMECG (□) in standard culture medium in the absence of cells. Catechins at a concentration of 50 μM were dissolved in 20 mL cellular medium without cells and incubated for 72 h in a CO_2 incubator. (B) Uptake of ECG (●) and TMECG (□) by Caco-2 cells. Caco-2 cells were grown until reaching 90% confluence, and then 20 mL of cellular medium containing 50 μM catechins and 0.2 mM ascorbic acid was added. For both stability and the cellular uptake experiments, 1 mL-aliquots of culture medium were taken at specified times and frozen in liquid N_2 until catechin concentration determination. The values presented are the mean percentage determined from five independent experiments ± SD.

Figure 2. (A) Caco-2 viability after treatments with TMECG and tea catechins and respect to a control experiment (100% viability) with no compound added. Cells were treated for 6 days with 25 μM of each compound. The values presented are the mean percentage determined from three independent experiments ± SD. (B) Morphological aspect (magnification × 800) of untreated SkMel-28 cells (control) compared with those subject to 6-days' treatments with 20 μM TMECG.

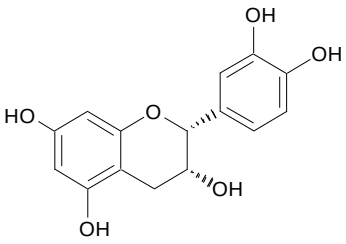
Figure 3. (A) Antioxidant activity of TMECG and tea catechins expressed as TEAC and determined as detailed in the Experimental Section. (B) Prooxidant activity of TMECG and tea catechins determined by their NADPH oxidation capacity in the absence or the presence of SOD. The rate of the oxidation of NADPH (0.1 mM) was calculated in the presence of 50 μM catechins in sodium-phosphate buffer at pH 7.4, 37 ºC. The effect of

SOD on catechin-dependent NADPH oxidation was studied in the presence of 10 μg/mL of enzyme.

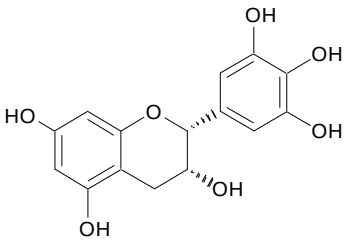
Figure 4. (A) Relative human DHFR inhibition by TMECG and tea catechins (all compounds at 100 μM) with respect to a control experiment with no compound added. Each bar represents the mean of five separate experiments. (B) Titration fluorescence experiments for the binding of TMECG and EGC to human DHFR. The points are experimental (after correction for enzyme dilution) and the lines are best-fit theoretical curves. The enzyme concentration was 0.1 μM. (C) View of TMECG modeled into the folate-binding site of human DHFR. Atoms of the TMECG ligand are colored green. Four different ligands from human and chicken DHFR crystal structures were used to define a binding envelope; 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 PDB structure files were used; 1DR1 (biopterin), 1S3V (TQD), 1S3W, and 1DLR.

Figure 5. Time-dependent expression of DHFR, TS and MTHFR in SkMel-28 cells treated with 20 μM TMECG. Data were obtained by real time PCR and compared with the relative expression of DHFR, TS and MTHFR in HeM cells (zero relative gene expression). Zero time represents the level of DHFR in untreated SkMel-28 cells. Differences between times were statistically significant ($P < 0.025$).

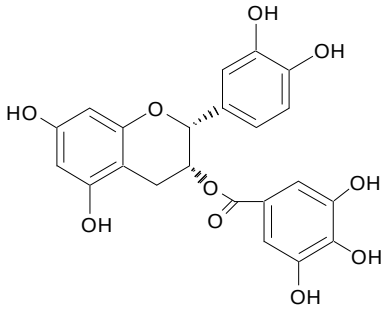
Chart 1



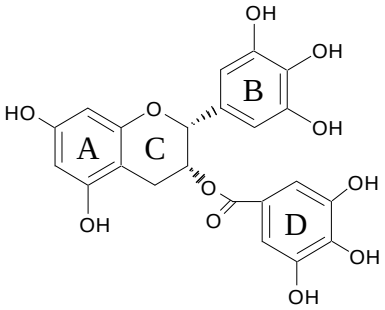
(-)-Epicatechin (EC)



(-)-Epigallocatechin (EGC)

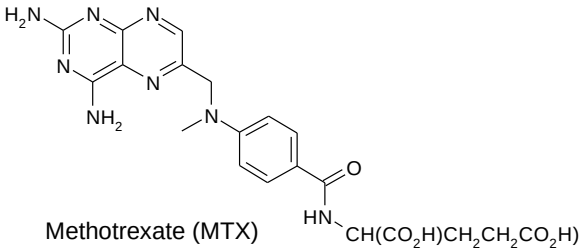


(-)-Epicatechin-3-gallate (ECG)

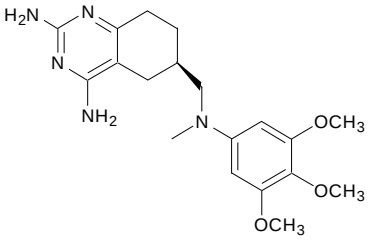


(-)-Epigallocatechin-3-gallate (EGCG)

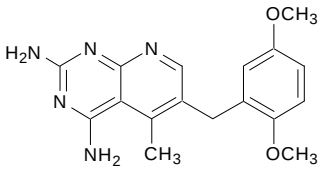
Chart 2



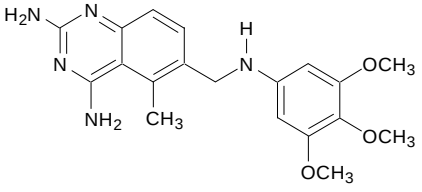
Methotrexate (MTX)



(R)-6-{[methyl-(3,4,5-trimethoxyphenyl)-amino]methyl}-5,6,7,8-tetrahydroquinazoline-2,4-diamine (TQD)

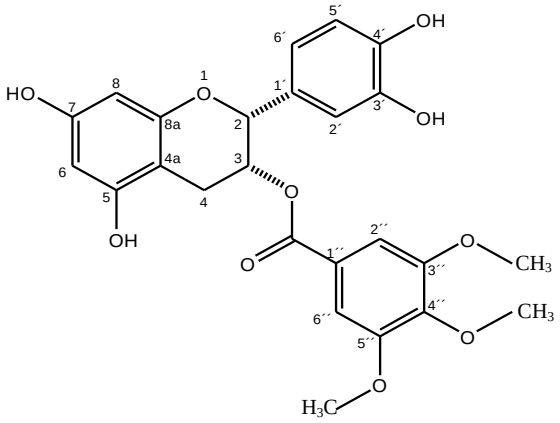


Piritrexim (PTX)



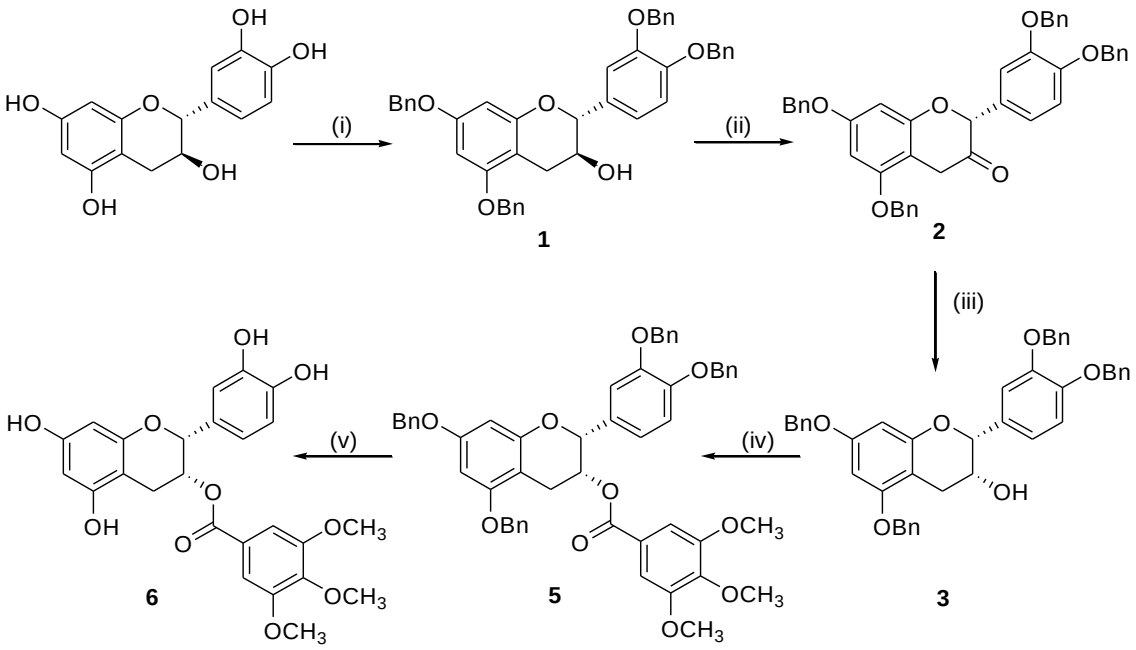
Trimetrexate (TMX)

Chart 3



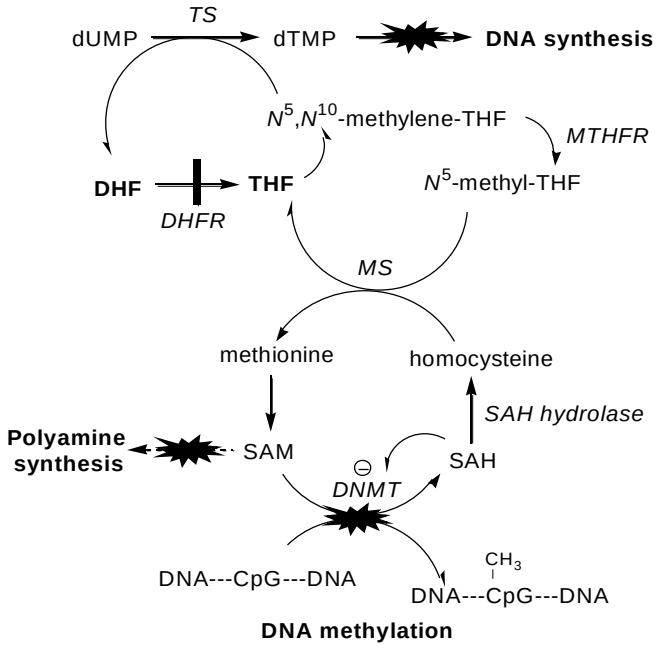
3-*O*-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG)

Scheme 1[a]



[a] Reagents and conditions: (i) benzyl bromide, K_2CO_3, DMF, -15 ºC to rt; (ii) Dess-Martin periodinane, moist CH_2Cl_2, rt; (iii) L-selectride®, *n*-Bu_4NCl, THF, -78 ºC; (iv) 3,4,5-trimethoxybenzoyl chloride, **4**, CH_2Cl_2, DMAP, -15 ºC to rt; (v) H_2, 20% $Pd(OH)_2/C$, THF/MeOH, rt.

Scheme 2


TS
dUMP
dTMP
DNA synthesis
N^5,N^{10}-methylene-THF
MTHFR
DHF
THF
N^5-methyl-THF
DHFR
MS
methionine
homocysteine
SAH hydrolase
Polyamine synthesis
SAM
SAH
DNMT
DNA---CpG---DNA
CH3
DNA---CpG---DNA
DNA methylation

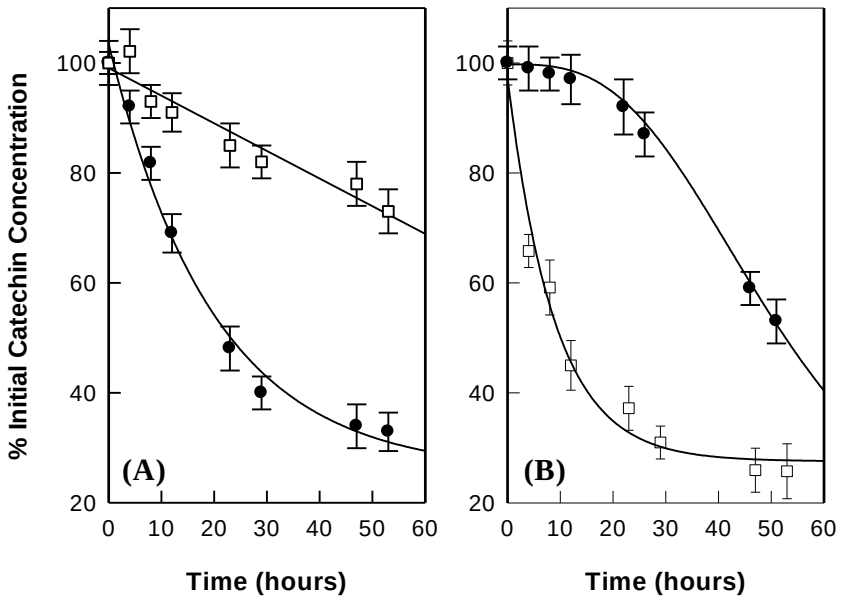


Figure 1. Sánchez-del-Campo et al

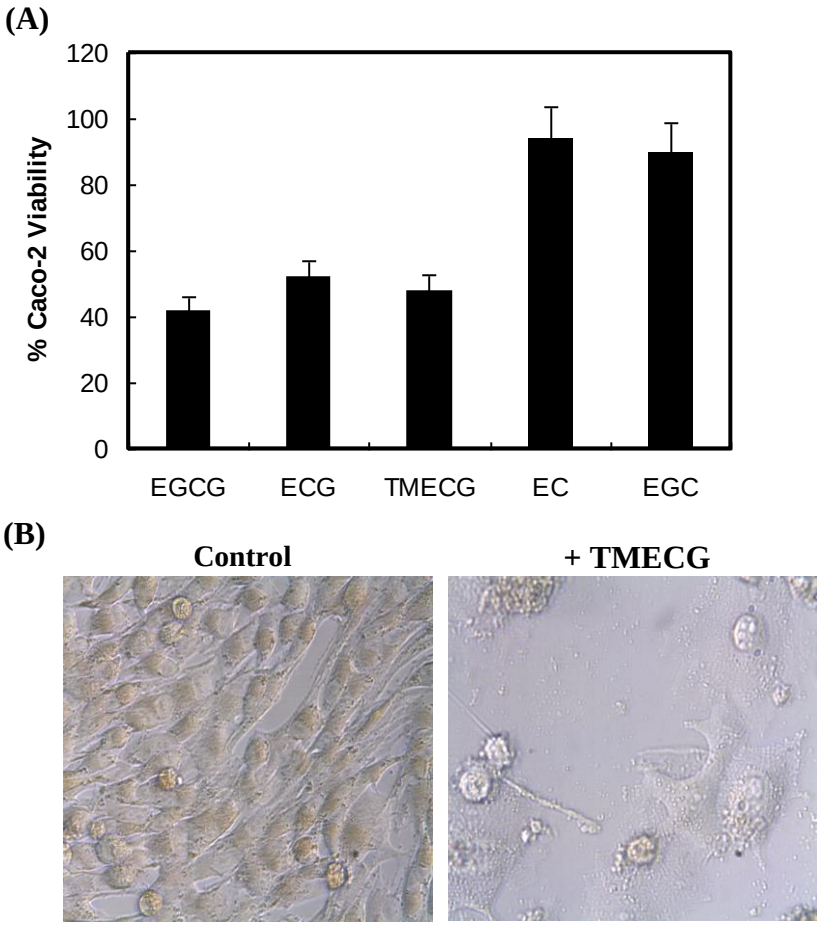


Figure 2. Sánchez-del-Campo et al

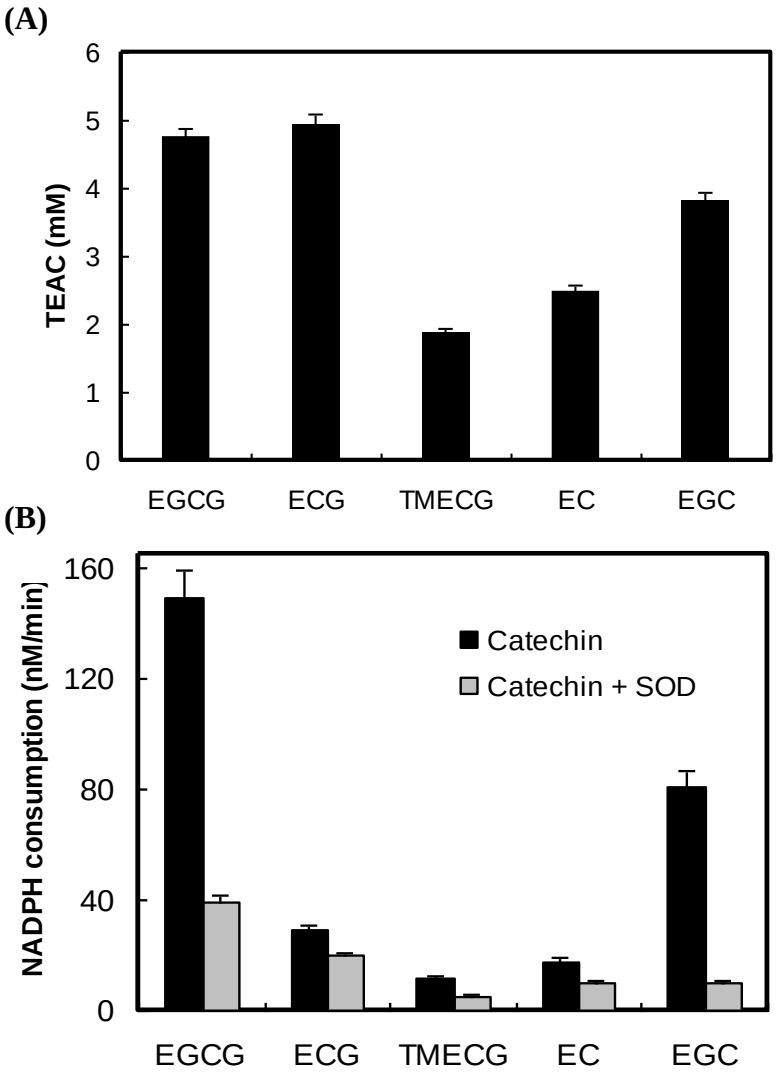


Figure 3. Sánchez-del-Campo et al.

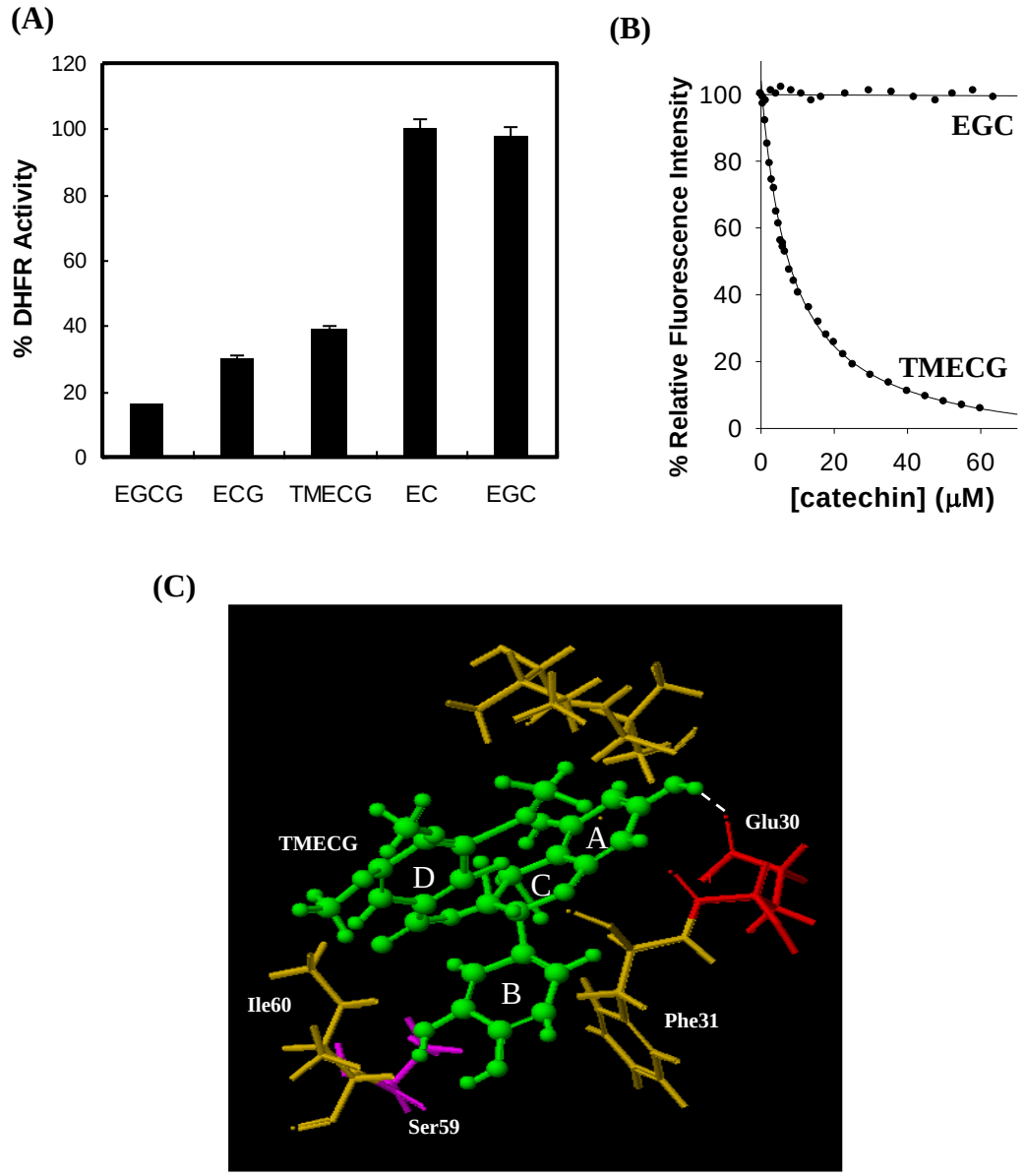


Figure 4. Sánchez-del-Campo et al.

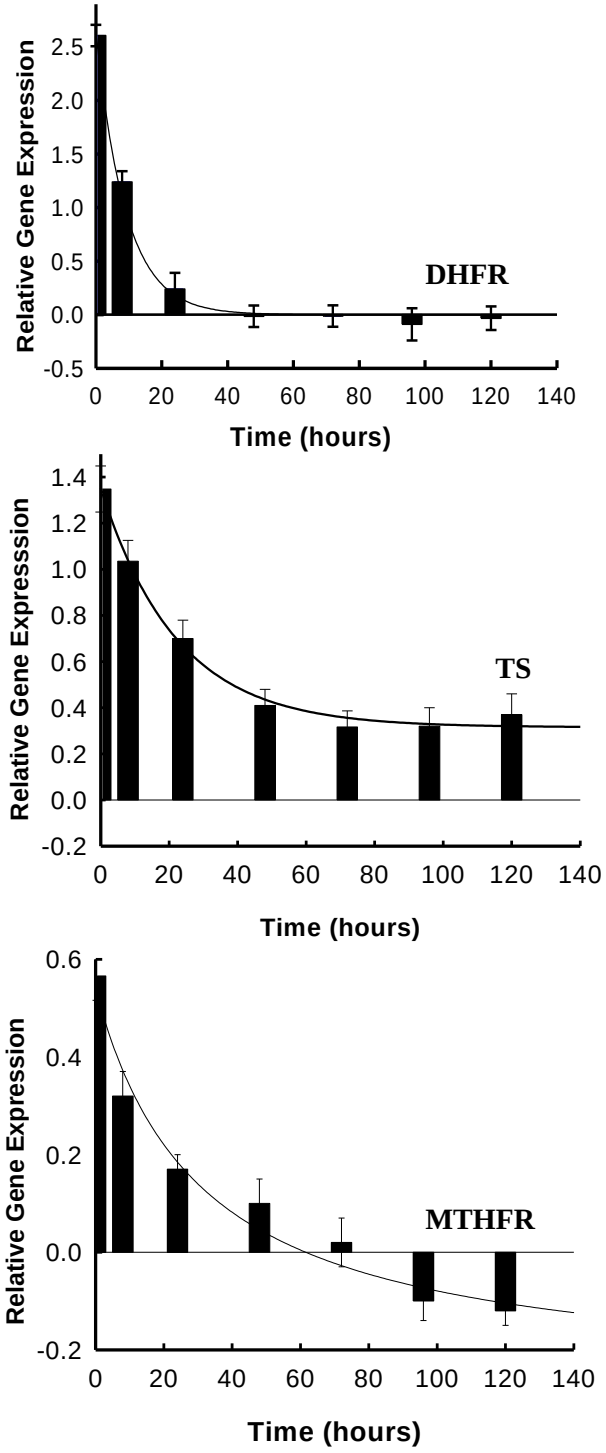


Figure 5. Sánchez-del-Campo et al

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Synthesis and Biological Activity of a 3,4,5-Trimethoxybenzoyl Ester Analogue of Epicatechin-3-Gallate

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