Effects of folate cycle disruption by the green tea polyphenol epigallocatechin-3-gallate

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Abstract

We demonstrate that the tea polyphenol, epigallocatechin-3-gallate, is an efficient inhibitor of human dihydrofolate reductase. Like other antifolate compounds, epigallocatechin-3-gallate acts by disturbing folic acid metabolism in cells, causing the inhibition of DNA and RNA synthesis and altering DNA methylation. Epigallocatechin-3-gallate was seen to inhibit the growth of a human colon carcinoma cell line in a concentration and time dependent manner. Rescue experiments using leucovorin and hypoxanthine-thymine medium were the first indication that epigallocatechin-3-gallate could disturb the folate metabolism within cells. Epigallocatechin-3-gallate increased the uptake of [^{3}H]-thymidine and showed synergy with 5-fluorouracil, while its inhibitory action was strengthened after treatment with hypoxanthine, which indicates that epigallocatechin-3-gallate decreases the cellular production of nucleotides, thus, disturbing DNA and RNA synthesis. In addition to its effects on nucleotide biosynthesis, antifolate treatment has been linked to a decrease in cellular methylation. Here, we observed that epigallocatechin-3-gallate altered the *p16* methylation pattern from methylated to unmethylated as a result of folic acid deprivation. Finally, we demonstrate that epigallocatechin-3-gallate causes adenosine to be released from the cells because it disrupts the purine metabolism. By binding to its specific receptors, adenosine can modulate different signalling pathways. This proposed mechanism should help us understand most of the molecular and cellular effects described for this tea polyphenol.

1. Introduction

Folic acid is a water-soluble B vitamin that plays a crucial role in DNA synthesis, its stability, integrity and repair. The active form of folic acid is 5,6,7,8-tetrahydrofolate (THF), which is formed in the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) by the enzyme dihydrofolate reductase (DHFR). THF serves as the principal component in folate metabolism, where it acts as a carrier of one-carbon units in its various cofactor forms. The synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP) is catalyzed by thymidilate synthase (TS) and uses 5,10-methylene-THF as the source of the methyl group of dTMP and as the reductant. This reaction requires DHF to be reduced by DHFR to THF. Thus, TS coupled with DHFR forms a crucial link responsible for the synthesis of dTMP and hence DNA. Folic acid may also modulate DNA methylation, which is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, in chromosomal modifications, and in the development of mutations (Kim, 2003; Widschwendter & Jones, 2002).

Inhibitors of folic acid metabolism, also called antifolates, have provided important agents useful in cancer chemotherapy and antibiotics (Berman & Werbel, 1991). Based on the observation that classical (MTX) and nonclassical (TMP) antifolate compounds present similar chemical structures to some tea polyphenols, we hypothesised that tea catechins could well inhibit DHFR activity. Recently, we have shown that ester-bonded gallate catechins from green tea, such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), are potent *in vitro* inhibitors of several DHFRs at concentrations found in the serum and tissues of green tea drinkers (0.1–1.0 μM) (Navarro-Perán, Cabezas-Herrera, García-Cánovas, Durrant, Thorneley, & Rodríguez-

López, 2005a; Navarro-Perán, Cabezas-Herrera, Hiner, Sadunishvili, García-Cánovas, & Rodríguez-López, 2005b; Navarro-Martínez, Navarro-Perán, Cabezas-Herrera, Ruiz-Gómez, García-Cánovas, & Rodríguez-López, 2005). Interestingly, a recent publication shows that ECG and EGCG efficiently inhibit cellular folic acid uptake in Caco-2 cells (Alemdaroglu, Wolffram, Boissel, Closs, Spahn-Langguth, & Langguth, 2007). These results suggested that catechins could act as antifolate compounds in the same way as MTX. Additional evidence that supports this observation can be obtained by examination of the related bibliography. Green tea has found similar clinical applications as antifolate compounds in the treatment of cancer, microbial and fungal infections, Crohn's disease, psoriasis or in chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis (Alic, 1999; Rasheed & Haider, 1998; Sueoka et al., 2001), and, surprisingly, EGCG and MTX show similar cellular and molecular effects against tumour development and progression (Seitz, 1999; Yang, Chung, Yang, Li, Meng, & Lee, 2000). To check our hypothesis that EGCG could act as an antifolate compound, we investigated the disruption of folate metabolism induced by this compound in Caco-2 cell monolayer, which acted as a model of the human intestinal epithelium. Colon cancer cells are highly sensitive to antifolate compounds (Singh, Fouladi-Nashta, Li, Halliday, Barret, & Sinclair, 2006) and the results obtained in this model could be of importance for understanding the epidemiological data that correlate the ingestion of green tea with the low risk of suffering gastrointestinal cancer (Mu et al., 2005).

2. Materials and methods

2.1. Materials

Tea polyphenols, EGCG (>95%), ECG (>98%), epigallocatechin (EGC; >98%) and epicatechin (EC; >98%), were purchased from Sigma Chemical Co. (Madrid, Spain). Green tea extract was obtained from fresh tea (*Camellia sinensis*) leaves as described elsewhere (Navarro-Perán et al., 2005b). Recombinant human DHFR (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 (Smith, Patrick, Stone, Phillips, & Burchall, 1979). DHF (90%) was obtained from Aldrich Chemical Co. (Madrid, Spain). NADPH, MTX, α,β-methylene adenosine-5'-diphosphate (APCP), thymine, hypoxanthine, 5-fluorouracil (5-FU), leucovorin (5-formyl-THF) and adenosine deaminase (ADA) were from Sigma.

2.2. DHFR assays

The activity of DHFR was determined at 25ºC by following the decrease in the absorbance of NADPH and DHF at 340 nm (ε = 11800 $M^{-1}cm^{-1}$) (Stone & Morrison, 1986) in a Perkin-Elmer Lambda-35 spectrophotometer. The slow development of catechin inhibition was determined by continuously monitoring the disappearance of NADPH and DHF after initiating the reaction by adding DHFR. Progress curves were obtained under ordered conditions ([NADPH] >> [DHF]). The calculation of rHDHFR inhibition constants by EGCG was performed as described elsewhere (Navarro-Perán et al., 2005b).

2.3. Fluorescence studies

The dissociation constant for the binding of EGCG to free rHDHFR was determined at 25ºC by fluorescence titration in an automatic-scanning Perkin-Elmer LS50B spectrofluorimeter 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 after addition of microliter volumes of a concentrated stock solution of ligand. Fluorescence emission spectra were recorded when rHDHFR fluorescence was excited at 290 nm.

2.4. Cell culture and treatments

Caco-2 cells were purchased from the ATCC (Rockville, USA) and were cultured in DMEM (Gibco, Barcelona, Spain) supplemented with 10% fetal calf serum (FCS) (Gibco), 2 mM L-glutamine (Gibco), 100 μg/ml of penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 1 mM piruvate and non-essential aminoacids solution (Gibco) at 37ºC in a humidified atmosphere 95% air-5% CO_2. Caco-2 cells were plated at a density of 1×10^6 cells/75 cm^2 flask and treated when they had reached 70% confluence. Cell injury was evaluated by a colorimetric assay for mitochondrial function using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. 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. Co-treatments of Caco-2 cells with EGCG and other reagents were carried out by supplementing medium cultures with HT medium (Sigma), thymine (0.1 mM), hypoxanthine (0.1 mM), leucovorin (0.1 mM), adenosine (10 μM), APCP (50 μM), 5-FU (50 μM) or ADA (10 μg/ml).

2.5. DNA fragmentation assay

DNA extraction from Caco-2 cells was performed as described elsewhere (Manna, Banerjee, Mukherjee, Das, & Panda, 2006). PBS-washed cells were resuspended in DNA extraction buffer (50 mM Tris, pH 8.0, 20 mM Na-EDTA, 10 mM NaCl, 1% [w/v] SDS, and 20 μg/ml RNase A) and incubated at 37ºC for 1 h, followed by proteinase K (100 μg/ml) treatment at 65ºC for 1 h. The DNA was extracted once with equal volume of buffer saturated phenol and once with chloroform:isoamyl alcohol mixture [24:1] followed by ethanol precipitation. Then DNA was dissolved in TE (10 mM Tris, pH 7.4, 1 mM EDTA) and analyzed by 1.5% agarosa gel electrophoresis.

2.6. Caspase-3 activity assay

Caspase-3 activity in the Caco-2 cells was measured using the caspase-3 colorimetric activity assay kit (Sigma). Briefly, the cells were washed with cold PBS and lysed with cell lysis buffer included in the kit. The cell lysates were centrifuged (10,000*g* for 5 min at 4ºC), and the supernatants were collected. Equal amounts of protein (100 μg), 30 μg of colorimetric caspase-3 substrate (acetyl-Asp-Glu-Val-Asp *p*-Nitroanilide; Ac-DVED-*p*NA) and assay buffer were added to each reaction mix, which were then incubated for 4 h at 37ºC. Caspase-3 activity was determined by measuring the absorbance at 405 nm. One unit of caspase-3 activity was expressed as 1 pmol of *p*NA formed per hour, and its specific activity was that unit divided by micrograms of protein. Caspase-3 inhibitor (Ac-DVED-CHO) was used as negative control.

2.7. Thymidine assay

Caco-2 cells were plated in 24-well trays at 4000 cells/well in 1 ml of DMEM supplemented as above (basal medium) and containing 100 μM hypoxanthine. Cultures were treated with or without EGCG or MTX, and 1 μCi of [^{3}H]-thymidine ([^{3}H]-Thy; 10Ci/mmol; American Radiolabeled Chemicals Inc., St Louis, MO, USA) was added to each well. After four days, cultures were washed 3 times with ice-cold phosphate-buffered saline and twice with cold 5% trichloroacetic acid (TCA) and solubilized with 0.1 N NaOH containing 1% sodium dodecyl sulfate. Radioactivity was determined by liquid scintillation counting (Winspectral 1414, Perkin-Elmer, Wellesley, MA, USA).

2.8. p16 methylation-specific PCR (MSP)

DNA was extracted from cells at each time and in each condition using the DNeasy tissue kit (Quiagen, Valencia, CA). Methylation-specific PCR (MSP) was performed using the CpGenome DNA modification kit, while the CpG WIZ p16 amplification kit (Chemicon Int., Temecula, CA) permitted sensitive detection of altered DNA. Amplification was carried out using an Eppendorf Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany).

2.9. NF-κB assays

To study the effect of EGCG on the activation of NF-κB in Caco-2 cells, nuclear fractions were prepared as described elsewhere (Ahmad, Gupta, & Mukhtar, 2000). Electrophoretic mobility shift assays (EMSA) were performed with a gel shift assay system kit from Promega (Madison, WI, USA), according to the manufacturer's protocol.

2.10. Western blot analysis

To study the effect of EGCG on adenosine and NF-κB related proteins, cytosolic fractions were obtained as described elsewhere (Ahmad et al., 2000). For immunoblot analysis, 25-50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated for 1 h in blocking solution (Tris-buffered saline containing 1% Tween 20 and 5% non-fat dry milk) and further incubated, overnight at 4ºC, with appropriate primary antibodies. IκBα was detected with primary IκBα antibodies (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA). A_1 (1:500), A_{2A} (1:500), A_{2B} (1:500) and A_3 (1:500) adenosine receptor antibodies were purchased from Santa Cruz Biotechnology. The membranes were then washed with blocking solution and incubated for 2 h with anti-mouse, anti-rabbit or anti-goat secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were detected by chemiluminiscence using an ECL Plus detection kit (GE Healthcare, Barcelona, Spain).

2.11. Confocal imaging

Caco-2 cells were cultured over 35 mm glass bottom microwell dishes to 50% confluence and treated with vehicle, EGCG or EGCG plus leucovorin. After washing in PBS, the cells were incubated for 1h with PBS containing primary adenosine receptor antibodies (1:25 for each: Santa Cruz Biotechnology) followed by incubation (1 h) with PBS containing secondary antibodies (Alexa Fluor Dyes, Invitrogen, Barcelona, Spain). Cells were analyzed by confocal laser scanning microscopy (Leica TCS 4D; Leica Microsystems GmbH, Wetzlar, Germany) at 750-fold magnification.

3. Results

3.1. EGCG is a slow-binding inhibitor of human dihydrofolate reductase

Green tea extracts containing significant amounts of tea catechins strongly inhibited the activity of rHDHFR. In order to detect which components of these extracts were responsible for such inhibition, rHDHFR activity was assayed in the presence of EC, EGC, ECG or EGCG. The results showed that both ECG and EGCG were potent inhibitors of the human enzyme, while polyphenols lacking the ester bound gallate moiety (*e.g.* EGC and EC) did not inhibit rHDHFR. The effective binding of EGCC to free rHDHFR was determined by following the decrease in enzyme fluorescence that occurs after formation of the enzyme-inhibitor complex (Fig. 1A). When rHDHFR fluorescence is excited at 290 nm its emission spectrum shows a maximum at 340-350 nm. The binding of EGCG quenches this fluorescence and the data showed that the dissociation constant of the enzyme-inhibitor complex (0.92 ± 0.03 μM) was lower than that for NADPH (10 μM). Similar results were obtained for ECG (data not shown); however, EC did not modify the emission spectra of rHDHFR (Fig. 1B). 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 the bovine enzyme (Navarro-Perán et al., 2005a; Navarro-Perán et al., 2005b). When rHDHFR activity was continuously assayed after addition of the enzyme to assay mixtures containing EGCG and enzyme substrates (NADPH and DHF), the resulting progress curves displayed time-dependent decreases in the reaction rates and finally attained steady-state velocities which varied as a function of inhibitor concentration (Fig. 2). The origin of this slow-binding inhibition is the formation of a slow dissociation ternary complex by the reaction of NADPH with the enzyme-inhibitor complex (Navarro-Perán et al., 2005a). The corresponding data

analysis of Fig. 2 indicates that EGCG inhibited rHDHFR with an overall inhibition constant of 33.0 ± 0.2 nM. These kinetic results show that EGCG is an efficient inhibitor of rHDHFR although softer than MTX, for which the inhibition constant is in the picomolar range. EGCG should therefore be assigned to the class of 'soft' DHF analogue inhibitors of DHFR (Graffner-Nordberg, Kolmodin, Aqvist, Queener, & Hallberg, 2001).

3.2 Folinic acid and hypoxanthine-thymine reverse the effect of EGCG on cell growth

To determine whether EGCG inhibits DHFR activity *in vivo*, a human colorectal cancer cell line was incubated with EGCG. This catechin was seen to significantly inhibit Caco-2 growth in a concentration- and time-dependent manner (Figs. 3A and 3B), and many damaged cells were observed after 3 days' treatment with 20 μM EGCG (Fig. 3C). Morphological changes included cell shrinkage, loss of cell-cell contact and the fragmentation of plasmatic and nuclear membranes. To investigate whether treatment with EGCG induced apoptosis in Caco-2, cells were treated with 50 μM EGCG for 72 h. DNA fragmentation, which is typical of apoptosis, was visible after treatment (Fig. 3D). Another feature of apoptotic cell death, the activation of caspase-3, was evaluated by colorimetric activity assay. The cells treated with EGCG showed significantly higher caspase-3 activity, which was inhibited by Ac-DVED-CHO (Fig. 3E). Clear evidence of the antifolate activity of EGCG against colon cancer cells was obtained from 'rescue' experiments using folinic acid (leucovorin). The administration of exogenous reduced folates, such as leucovorin, effectively prevents antifolate cytotoxicity in mammalian cells. Figs. 3B-3E show that the effects of EGCG on cell growth, morphology and induced apoptosis were effectively reversed by co-treatment

with leucovorin. Growing the cells in a medium containing hypoxanthine-thymine (HT medium) also significantly attenuated the effects of EGCG (Fig. 3B). It is well known that cells expressing hypoxanthine-guanine phosphoribosyltransferase, an enzyme essential for the recycling of purine nucleotides, can survive in the presence of antifolates in HT medium.

3.3. EGCG disturbs DNA and RNA synthesis in Caco-2 cells.

To determine the effects of EGCG on DNA synthesis we studied the extent to which [^{3}H]-Thy is incorporated in the DNA of cells treated with EGCG or MTX. To avoid the inhibition of DNA and RNA synthesis cold hypoxanthine was added to the cell medium. The effects of antifolates on the incorporation of [^{3}H]-Thy in DNA is well known (daCosta, Rothenberg, Kamen, 1972). By inhibiting DHFR, these drugs block the *de novo* methylation of dUMP to TMP. Thus, the addition of MTX to cancer cells enhances the incorporation of exogenous [^{3}H]-Thy into DNA (Table 1). A similar effect was observed by treating the cells with EGCG (Table 1), demonstrating that this drug disturbs DNA synthesis. Additional confirmation of the antifolate activity of EGCG was obtained in synergy experiments using 5-FU. This fluoropyrimidine is an antimetabolite drug and is widely used for the treatment of cancer, particularly colorectal cancer. 5-FU exerts its anticancer effects by inhibiting TS and incorporating its metabolites into RNA and DNA. The synergistic effect between antifolates and TS inhibitors is well known and modulation strategies, such as co-treatment with leucovorin and MTX, have been developed to increase the anticancer activity of 5-FU (Fernandes & Bertino, 1980). The identification of EGCG as an antifolate drug encouraged us to investigate the effects of combining both EGCG and 5-FU. The synergic effect of both drugs on Caco-2 growth

is apparent in Fig. 4A. The effect of EGCG on RNA synthesis was demonstrated by co-treatment with EGCG and hypoxanthine or thymine. Although a single treatment with thymine showed a slight reversion of EGCG cell growth inhibition, hypoxanthine had the opposite effect and enhanced EGCG cytotoxicity (Fig. 4B). These results are consistent with DHFR being the site of action of EGCG. By inhibiting DHFR, antifolates deplete cellular stores of reduced folates, resulting in the inhibition of DNA and RNA synthesis. The inhibition of RNA synthesis arrests cells in the G1 phase of the cell cycle, preventing such cells from entering the S phase and rendering them insensitive to antifolates. It has been described that hypoxanthine increases MTX cytotoxicity by maintaining RNA synthesis, allowing cells that might be arrested in G1 to progress into the S phase, which is associated with apoptotic cell death (Fairchild, Maybaum, & Straw, 1988).

3.4. Inhibition of DNA methylation and reactivation of tumour suppressor p16 expression by EGCG are mediated by folate cycle disruption

As observed in a human esophageal squamous carcinoma cell line (Fang et al., 2003), *p16* had hypermethylation status in Caco-2 cells (Fig. 5). Treatment of this cancer cell line with 20 μM EGCG for 5 days showed that the *p16* methylation pattern changed from methylated to unmethylated (Fig. 5). Western blot analysis indicated that p16 protein expression increased in cells subject to this treatment (Fig. 5). To ascertain whether the DNA hypomethylation induced in cancer lines by EGCG in our laboratory and those of others was due to the inhibition of DNMT or to a reduction in the folate pool associated with DHFR inhibition, we designed and carried out two series of experiments. In the first, we studied the methylation status of *p16* in Caco-2 cells

treated with MTX, observing that hypermethylation could be reversed (Fig. 5). In the second series of experiments, we checked whether *p16* hypomethylation induced by EGCG or MTX could be reversed by growing the cells in a medium containing leucovorin. In both cases *p16* was found to have hypermethylation status (Fig. 5).

3.5. Adenosine is required for EGCG cytotoxicity

Next, we evaluated the extent to which the blockage of the purine biosynthesis pathway by EGCG and the subsequent release of adenosine contributes to the inhibition of Caco-2 growth. Although adenosine was seen to have little inhibitory effect on cell growth, this effect could be significantly enhanced by co-treatment with EGCG, both compounds acting synergistically (Fig. 6A). To ascertain whether EGCG suppressed Caco-2 growth by enhancing adenosine release, we designed a series of experiments aimed at depleting extracellular adenosine. For this, Caco-2 cells were treated with EGCG in the presence of ADA or APCP, a competitive inhibitor of ecto-5' nucleotidase, and it was seen that both treatments were able to completely reverse the EGCG effect (Fig. 6A). These results suggest that the inhibitory effect of EGCG on Caco-2 growth is mediated by adenosine.

3.6. EGCG induces A_3AR expression and differentially modulates the activation of NF-κB in colon cancer cells

To further understand the role of adenosine in this process we investigated the effect of EGCG on the constitutive activation of NF-κB in Caco-2 cells. It has been show that NF-κB is constitutively activated in human colorectal carcinoma tissue (Yu,

Yu, Yu, Luo, Xu, & Li, 2004). Here we show that EGCG differentially modulates NF-κB in a time-dependent manner. After treatment of Caco-2 cells with 20 μM EGCG for one day, NF-κB was highly activated compared with an untreated control (Fig. 6B). This activation was effectively reversed by co-treatment with leucovorin or APCP (Fig. 6B). After the same period of time (one day), adenosine alone or in combination with EGCG showed a similar effect, enhancing NF-κB activation (Fig. 6B). By binding to A_1AR, adenosine activates NF-κB though a pathway that involves the decrease of cAMP, the liberation of calcium from endoplasmic reticulum and activation of the PKC pathway (Basheer, Rainnie, Porkka-Heiskanen, Ramesh, & McCarley, 2001). Although adenosine had the same effect after longer treatments (*e.g.* three days), the lack of NF-κB activation by EGCG alone or in combination with adenosine (Fig. 6B) cannot be explained by the participation of this adenosine receptor. To understand the involvement of different types of adenosine receptors in this process their levels in the plasmatic membranes of Caco-2 cells and their response to EGCG treatment were analyzed by both Western blot analysis and confocal microscopy. It was seen that EGCG differentially modulated the expression of adenosine subtype receptors. Although A_1AR and A_2AR were not significantly increased after 3 days' treatment with 20 μM EGCG, A_3AR did show a significant increase after this time (Fig. 6C). This increase in A_3AR was reversed by co-treatment of the cells with EGCG plus leucovorin (Fig. 6C). These findings clearly indicate that the A_3AR signaling pathway is the predominant event in more prolonged treatments with EGCG, since its action was able to suppress the constitutive activation of NF-κB in colon cancer cells and potentate the inhibitory action of EGCG in the presence of adenosine (Figs. 6A and 6B).

4. Discussion

Green tea has shown anti-cancer properties in many animal tumour and cell culture systems, as well as in epidemiological studies. On the basis of these findings, multiple mechanisms for the action of EGCG have been proposed (Jung & Ellis, 2001; Lambert & Yang, 2003). However, no mechanism that covers all the cellular and molecular effects found for this compound has been postulated. The data presented here clearly show how EGCG inhibits human DHFR activity and how it disrupts the folate cycle. Disruption of the cell folate cycle by EGCG may explain many of the molecular and cellular effects described for this tea polyphenol (Jung & Ellis, 2001; Lambert & Yang, 2003) because antifolates exert their action by disturbing the nucleic acid metabolism of cancer cells, including its synthesis, methylation and stability (Scheme 1).

The efficacy of antifolates in treating cancer is widely attributed to their ability to reduce nucleotide synthesis. Antifolates treatment has also been linked to a decrease in cellular methylation (Scheme 1). DNA methylation patterns are frequently altered in human cancer, and include genome-wide hypomethylation as well as regional hypermethylation at CpG islands (Jones & Laird, 1999; Widschwendter & Jones, 2002). The CpG island methylated phenotype in colorectal cancer is characterized by the frequent and concurrent methylation of specific CpG sites, including those present in the promoter regions of *p16* and *hMLH1* (Ushijima & Sasako, 2004). P16 (CDKN2/MTS1), an inhibitor of the cyclin D-dependent protein kinase 4/6, is a cell cycle regulator involved in the inhibition of G1 phase progression (Serrano, Hannon, & Beach, 1993). Loss of function of *p16* results in higher cyclin D-dependent protein kinase activity and thus leads to the aberrant phosphorylation of retinoblastoma, which accelerates cell growth. Inactivation of *p16* by homozygous deletion or point mutation

is one of the most commonly observed aberrations in tumors, indicating that *p16* is a tumor suppressor gene. An alternative mechanism to explain the inactivation of *p16* is the aberrant methylation of CpG islands extending from the promoter region to exon 1, which silences transcription of this gene (Ushijima & Sasako, 2004). Recently, it has been shown that EGCG causes CpG demethylation and the reactivation of methylation-silenced genes though the inhibition of 5-cytosine DNA methyltransferase (DNMT) (Fang et al., 2003). However, folic acid deficiency has also been associated with perturbed DNA methylation (Kim, 2003; Niculescu & Zeisel, 2002). DNA is hypomethylated in the brains of rats fed a folate-deficient diet (Kim et al., 1997) or treated with MTX (Alonso-Aparte & Varela-Moreiras, 1996). Although it has been clearly demonstrated that DNMT is directly inhibited by EGCG *in vitro* (Fang et al., 2003), the reversal of *p16* hypomethylation by leucovorin in cancer cells treated with EGCG (Fig. 5) suggests that the DNA hypomethylation observed *in vivo* is related to the disruption of the folate cycle associated with DHFR inhibition. It is likely that low folate levels in cancer cells induced by EGCG treatment prevent hypermethylation and the silencing of these key genes, and so this inhibition could contribute to the prevention of carcinogenesis.

On the other hand, antifolates, by lowering THF cofactors, inhibit the purine synthesis pathway (Cutolo, Sulli, Pizzorni, Seriolo, & Straub, 2001; Majumdar & Aggarwal, 2001) (Scheme 1). This inhibition results in an increase of adenosine in the extracellular space. Adenosine is a potent endogenous regulator of a variety of physiological processes through specific receptors on cell surfaces and binds to four different types of G protein-coupled cell surface molecules, termed the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors, which have been found in many different cell types

(Fishman, Bar-Yehuda, Barer, Madi, Multani, & Pathak, 2001). For instance, it has been demonstrated that MTX suppresses NF-κB activation through the release of adenosine, which contributes to the anti-inflammatory, immunomodulatory, and antiproliferative properties of MTX (Majumdar & Aggarwal, 2001). Here we show that EGCG was able to modulate the constitutive activation of NF-κB in colon cancer cells and that adenosine is required for EGCG cytotoxicity (Fig. 6A). However, adenosine alone could not inhibit cell growth or suppress the constitutive activation of NF-κB. Importantly, it recently has been observed that MTX enhances its anti-inflammatory effect by up-regulating A_3AR expression (Ochaion et al., 2006). The induction of A_3AR was required for adenosine to act in EGCG-treated cells. Thus, in EGCG-treated cells, the binding of adenosine to A_3AR may inhibit NF-κB activation through a pathway that involves a decrease in cAMP, the inhibition of PKA and down-regulation of the PKB/Akt arm (Fishman et al., 2004). PKA and PKB/Akt use GSK-3β as a substrate, and upon phosphorylation, GSK-3β activity is inhibited. GSK-3β has been widely implicated in cell homeostasis, for its ability to phosphorylate a broad range of substrates, including β-catenin, a key component of the Wnt pathway. In normal cells, GSK-3β phosphorylates β-catenin, thereby inducing its ubiquitination and degradation by the proteosome system. However, in tumor cells, GSK-3β fails to phosphorylate β-catenin, leading to its accumulation in the cytoplasm. It then translocates to the nucleus, where it acts in concert with LEF-1 to induce the transcription of the cell cycle progression genes such as cyclin D1 and c-Myc (Fishman et al., 2004). Downregulation of these two oncogenes by EGCG has been widely described (Manna et al., 2006). Activation of A_3AR by EGCG treatment would suppress the activation of cyclin D1 and c-Myc, leading to cell cycle arrest and the induction of apoptosis (Chen, Shen, Hebbar,

Hu, Owuor, & Kong, 2003; Hwang et al., 2007). These findings together with the observed synergy between adenosine and EGCG against colon cancer cells indicate that combined therapies with EGCG and adenosine agonists may be a promising strategy for the treatment of certain types of cancer (Fishman et al., 2001).

In conclusion, we demonstrate that EGCG, by inhibiting DHFR and/or folic acid uptake (Alemdaroglu et al., 2007), can disturb the metabolism of this vitamin in Caco-2 cells. In any case, the effectiveness of EGCG as an antifolate drug would depend on its bioavailability at a particular organ. The presently observed effective concentration of EGCG on human DHFR (33 nM) is easily reached in the oral cavity after drinking tea, and perhaps in the stomach, esophagus and intestines. However, this concentration is higher than that reaching internal organs. Therefore, only a controlled human clinical trial will be able to truly explore this anticancer property and any side effects associated with the use of this compound.

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References

Ahmad, N., Gupta, S., & Mukhtar, H. (2000) Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor κB in cancer cells versus normal cells. *Arch. Biochem. Biophys.376*, 338-346.

Alemdaroglu, N. C., Wolffram, S., Boissel, J. P., Closs, E., Spahn-Langguth, H., & Langguth, P. (2007). Inhibition of folic acid uptake by catechins and tea extracts in Caco-2 cells. *Planta Med. 73,* 27-32.

Alic, M. (1999). Green tea for remission maintenance in Crohn's disease? *Am. J. Gastroenterol., 94*, 1710-1711.

Alonso-Aparte, E., & Varela-Moreiras, G. (1996). Brain folates and DNA methylation in rats fed a choline deficient diet or treated with low doses of methotrexate. *Int. J. Vitam. Nutr. Res.*, *66*, 232-236.

Basheer, R., Rainnie, D. G., Porkka-Heiskanen, T., Ramesh, V., & McCarley, R. W. (2001) Adenosine, prolonged wakefulness, and A_1-activated NF-κB DNA binding in the basal forebrain of the rat. *Neuroscience, 104*, 731-739.

Berman, E. M., & Werbel, L. M. (1991). The renewed potential for folate antagonists in contemporary cancer chemotherapy. *J. Med. Chem.*, *34*, 479-485.

Chen, C., Shen, G., Hebbar, V., Hu, R., Owuor, E. D., & Kong, A. N. (2003). Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis, 24*, 1369-1378.

Cutolo, M., Sulli, A., Pizzorni, C., Seriolo, B., & Straub, R. H. (2001). Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann. Rheum. Dis.*, 60, 729-735.

daCosta, M., Rothenberg, S. P., & Kamen, B. (1972). DNA synthesis in chronic myelogenous leukemia cells: comparison of results in cells containing folate binding factor to replicating cells without binder. *Blood*, *39*, 621-627.

Fairchild, C. R., Maybaum, J., & Straw, J. A. (1988). Enhanced cytotoxicity with methotrexate in conjunction with hypoxanthine in L1210 cells in culture. *Cancer Chemother. Pharmacol.*, *22*, 26-32.

Fang, M. Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, H., et al. (2003). Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.*, *63*, 7563-7570.

Fernandes, D. J., & Bertino, J. R. (1980). Fluorouracil-methotrexate synergy: enhancement of 5-fluorodeoxyridylate binding to thymidylate synthase by dihydropteroylpolyglutamates. *Proc. Natl. Acad. Sci. USA, 77*, 5663-5667.

Fishman, P., Bar-Yehuda, S., Barer, F., Madi, L., Multani, A. S., & Pathak, S. (2001). The A3 adenosine receptor as a new target for cancer therapy and chemoprotection. *Exp. Cell Res.*, *269*, 230-236.

Fishman, P., Bar-Yehuda, S., Ohana, G., Barer, F., Ochaion, A., Erlanger, A., et al. (2004) An agonist to the A_3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3β and NF-κB. *Oncogene, 23*, 2465-2471.

Graffner-Nordberg, M., Kolmodin, K., Aqvist, J., Queener, S. F., & Hallberg, A. (2001). Design, synthesis, computational prediction, and biological evaluation of ester soft drugs as inhibitors of dihydrofolate reductase from *Pneumocystis carinii*. *J. Med. Chem.*, *44*, 2391-2402.

Hwang, J. T., Ha, J., Park, I. J., Lee, S. K., Baik, H. W., Kim, Y. M., et al. (2007). Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett., 247*, 115-121.

Jones, P. A., & Laird, P. W. (1999). Cancer epigenetics comes of age. *Nat. Genet.*, *21*, 163-167.

Jung, Y. D., & Ellis, L. M. (2001). Inhibition of tumour invasion and angiogenesis by epigallocatechin-gallate (EGCG), a major component of green tea. *Int. J. Exp. Path.*, *82*, 309-316.

Kim, Y. I. (2003). Role of folate in colon cancer development and progression. *J. Nutr.*, *133*, 3731S-3739S.

Kim, Y. I., Pogribny, I. P., Basnakian, A. G., Miller, J. W., Selhub, J., James, S. J. et al. (1997). Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am. J. Clin. Nutr.*, *65*, 46-52.

Lambert, J. D., & Yang, C. S. (2003). Mechanisms of cancer prevention by tea constituents. *J. Nutr.*, *133*, 3262S-3267S.

Majumdar, S., & Aggarwal, B. B. (2001). Methotrexate suppresses NF-κB activation through inhibition of IκBα phosphorylation and degradation. *J. Immunol*., *167*, 2911-2920.

Manna, S. A., Banerjee, S., Mukherjee, S., Das, S., & Panda, C. K. (2006). Epigallocatechin gallate induced apoptosis in sarcoma180 cells in vivo: Mediated by p53 and inhibition in U1B, U4-U6 UsnRNAs expression. *Apoptosis, 11*, 2267-2276.

Mu, L. N., Lu, Q. Y., Yu, S. Z., Jiang, Q. W., Cao, W., You, N. C., et al. (2005). Green tea drinking and multigenetic index on the risk of stomach cancer in a Chinese population. *Int. J. Cancer*, *116*, 972-983.

Navarro-Martínez, M. D., Navarro-Perán, E., Cabezas-Herrera, J., Ruiz-Gómez, J., García-Cánovas, F., and Rodríguez-López, J. N. (2005). Antifolate activity of epigallocatechin gallate against *Stenotrophomonas maltophilia. Antimicrob. Agents Chemother.*, *49*, 2914-2920.

Navarro-Perán, E., Cabezas-Herrera, J., García-Cánovas, F., Durrant, M. C., Thorneley, R. N. F., & Rodríguez-López, J. N. (2005a). The antifolate activity of tea catechins. *Cancer Res.*, *65*, 2059-2064.

Navarro-Perán, E., Cabezas-Herrera, J., Hiner, A. N. P., Sadunishvili, T., García-Cánovas, F., & Rodríguez-López, J. N. (2005b). Kinetics of the inhibition of bovine liver dihydrofolate reductase by tea catechins: origin of slow-binding inhibition and pH studies. *Biochemistry*, *44*, 7512-7525.

Niculescu, M. D., & Zeisel, S. H. (2002). Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J. Nutr.*, *132*, 2333S-2335S.

Ochaion, A., Bar-Yehuda, S., Cohn, S., Del Valle, L., Perez-Liz, G., Madi, L. et al. (2006). Methotrexate enhances the anti-inflammatory effect of CF101 via up-regulation of the A3 adenosine receptor expression. *Arthritis Res Ther., 8*, R169.

Rasheed, A., & Haider, M. (1998). Antibacterial activity of *Camellia sinensis* extracts against dental caries. *Arch. Pharm. Res.*, *21*, 348-352.

Seitz, M. (1999). Molecular and cellular effects of methotrexate. *Curr. Opin. Rheumatol. 11*, 226-232.

Serrano, M., Hannon, G. J., & Beach, D. A. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature, 366*, 704-707.

Singh, R., Fouladi-Nashta, A. A., Li, D., Halliday, N., Barret, D. A., & Sinclair, K. D. (2006). Methotrexate induced differentiation in colon cancer cells is primarily due to purine deprivation. *J. Cell Biochem., 99*, 146-155.

Smith, S. L., Patrick, P., Stone, D., Phillips, A. W., & Burchall, J. J. (1979). Porcine liver dihydrofolate reductase: purification, properties, and amino acids sequence. *J. Biol. Chem., 254*, 11475-11484.

Stone, S. R., & Morrison, J. F. (1986). Mechanism of inhibition of dihydrofolate reductases from bacterial and vertebrate sources by various classes of folate analogues. *Biochim. Biophys. Acta, 869*, 275-285.

Sueoka, N., Suganuma, M., Sueoka, E., Okabe, S., Matsuyama, S., Imai, K., Nakachi, K., & Fujiki, H. (2001). A new function of green tea: prevention of lifestyle-related diseases. *Ann. N.Y. Acad. Sci.*, *928*, 274-280.

Ushijima, T., & Sasako, M. (2004). Focus on gastric cancer. *Cancer Cell*, *5*, 121-125.

Widschwendter, M., & Jones, P. A. (2002). DNA methylation and breast carcinogenesis. *Oncogene*, *21*, 5462-5482.

Yang, F., Chung, J. Y., Yang, G. Y., Li, C., Meng, X., & Lee ,M. J. (2000). Mechanisms of inhibition of carcinogenesis by tea. *Biofactors*, *13*, 73-79.

Yu, L., Yu, H., Yu, J., Luo, H., Xu, X., & Li, J. (2004) Nuclear factor-κB p65 (RelA) transcription factor is constitutively activated in human colorectal carcinoma tissue. *World J. Gastroenterol. 10*, 3255-3260.

Legend to Schemes

Scheme 1. General effects of antifolates on folic acid cycle. Abbreviations: AICAR; 5-aminoimidazole-4-carboxamide ribonucleotide; DHF, 7,8-dihydrofolate; DHFR, dihydrofolate reductase; DNMT, 5-cytosine DNA methyltransferase; dTMP, deoxythymidylate; dUMP, deoxyuridylate; FAICAR, formyl-AICAR; FGAR, formyl-GAR; GAR, glycinamide ribonucleotide; IMP, inosine 5'-monophosphate; MS, Methionine synthase; MTHFR, methylene-THF reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, 5,6,7,8-tetrahydrofolate; TS, thymidilate synthase.

Legend to Figures

Fig. 1. Titration fluorescence experiments for the binding of EGCG (A) and EC (B) to rHDHFR. The experiments were carried out 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 and 25 ºC in the presence catechin and rHDHFR (0.5 μM).

Fig. 2. Progress curves for the slow-binding inhibition of rHDHFR by EGCG. The experiments were carried out in the same buffer as Fig. 1 in the presence of NADPH (100 μM), DHF (8 μM), and rHDHFR (2.8 nM). Non-linear regression analysis of the progress curves to the following equation $P = v_s t + (v_0 - v_s)(1 - \exp(-k_{obs} t)) / k_{obs}$ (v_s, v_0 and k_{obs} represent the steady-state velocity, initial velocity and apparent first-order rate constant, respectively) yield the values of k_{obs} at different EGCG concentrations (inset). Results are the mean ± SD of triplicate determinations.

Fig. 3. Effects of EGCG on Caco-2 cells. (A) Time-dependent inhibition of Caco-2 growth by different concentrations of EGCG (μM): (●) 0; (○) 10; (■) 20; (□) 40 and (▲) 80. (B) Dose-dependent effect of EGCG and reversion experiments with leucovorin (L) and HT medium on cell growth after 3 days of treatment compared with untreated cells. The values presented are the mean percentage determined from three independent experiments ± SD. (C) Morphological aspect (magnification × 400) of untreated Caco-2 cells compared with those subject to 3-days treatments with 20 μM EGCG in the absence or the presence of leucovorin. (D) DNA laddering. Cellular DNA from Caco-2 cells was extracted and resolved by agarose gel electrophoresis. Lane 1, molecular marker; lane 2, Caco-2; lane 3, EGCG (50 μM) 3-days' treated Caco-2. (E) The effect of EGCG on the activity of caspase-3 in Caco-2 cells. Cells were treated for 3 days with EGCG in the absence or presence of leucovorin. Caspase-3 activity was determined in the absence or the presence of the inhibitor Ac-DEVD-CHO. The results are expressed as the mean ± SD of three cultures. The data are representative of duplicate activity

determinations. *P < 0.05, significantly different from non-treated group and **P < 0.05, significantly different from EGCG-treated group.

Fig 4. (A) Effect of EGCG (10 μM) and 5-FU (50 μM) alone or combined on the growth of Caco-2 cells after 24 and 72 h of treatment. The data are expressed assuming 100% growth for the untreated control. Bars represent the average growth for five individual experiments, and the error bars represent the SD of the data. *P < 0.001 compared with corresponding values for treatment without EGCG. (B) Effect of hypoxanthine (H, 100 μM) and thymine (T, 100 μM) on the growth inhibition of Caco-2 cells by EGCG (E; 20 μM) after 3 days of treatment. The data are expressed assuming 100% growth for the untreated control. Bars represent the average growth for three individual experiments, and the error bars represent the SD of the data.

Fig. 5. Effect of EGCG on the methylation status and expression of *p16* gene. (A) Alteration of methylation status of *p16* gene in Caco-2 cells after 5 days' treatment with 20 μM EGCG or 2 nM MTX in the absence (E or M, respectively) or the presence of leucovorin (EL or ML, respectively). The panel presents the methylation-specific bands (MSB). The amplified PCR products were subjected to electrophoresis in 2% agarose gel and then, the gel was stained with ethidium bromide and photographed. Negative controls for PCR were run under the same conditions without DNA. (B) Effect of EGCG treatments on the expression of p16 protein. Protein levels were determined with Western blot analysis using β-actin as an internal control. Band intensity was determined with densitometry and the values presented are the mean of three independent experiments; bars, ± SD.

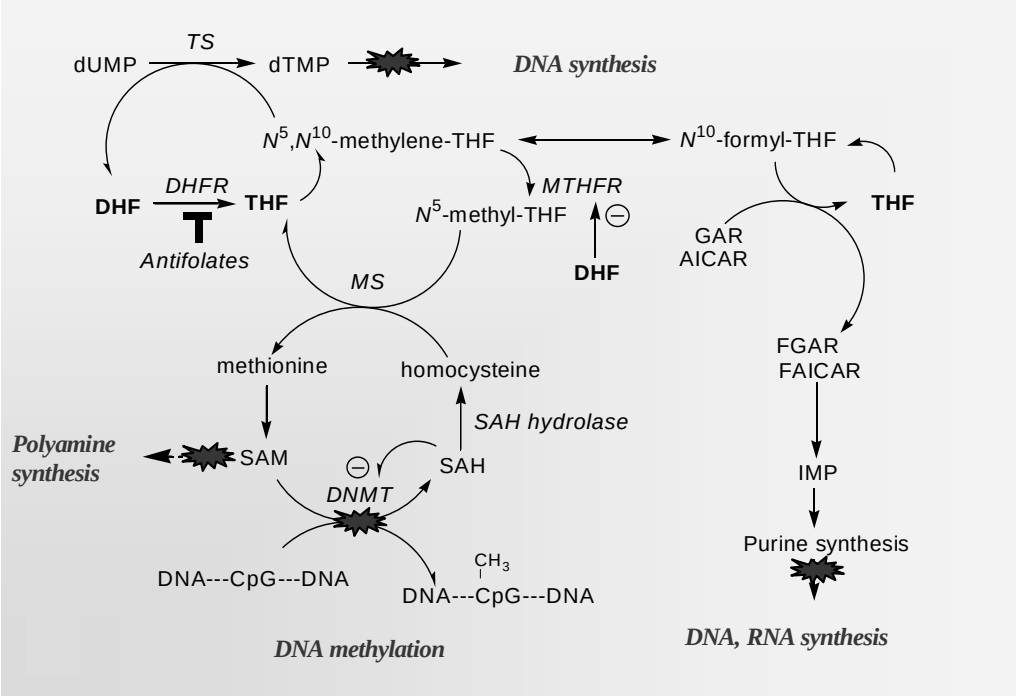
Fig. 6. (A) Effect of different treatment on Caco-2 growth. EGCG (E; 20 μM) or adenosine (Ado; 10 μM) alone or EGCG (20 μM) combinations with adenosine (10 μM), APCP (50 μM) and ADA (10 μg/ml). Growth was determined after 4 days' treatment and the data expressed assuming 100% growth for the untreated control. Bars represent the average growth for three independent experiments, and the error bars

represent the SD of the data. *P<0.0001 compared with corresponding values for individual treatments with EGCG or adenosine. (B) IκBα level in cytosolic fractions of Caco-2 cells subject to different 1- or 3-day treatments with EGCG (20 μM) or adenosine (10 μM) alone or EGCG (20 μM) combinations with leucovorin (L; 100 μM), adenosine (10 μM), APCP (50 μM) and ADA (10 μg/ml). The data shown here are from a representative experiment repeated three times with similar results. (C) NF-κB activation in the some conditions as detailed in the section B of this Figure. The data shown here are from a representative experiment repeated three times with similar results. (D) Florescence confocal microscopy of Caco-2 after 3 days' treatment with vehicle only (NT), or 20 μM EGCG in the absence (E) or presence of leucovorin (EL). Induction folds of A_3AR calculated by both florescence confocal microscopy (three separate experiments) and western blot analysis (five separate experiments). Bars represent the means of the eight experiments carried out by both techniques ± SD.

Table 1 – Effect of MTX and EGCG on thymidine incorporation in Caco-2 cells

Addition	[^{3}H]Thy incorporated (dpm)[a]	Cell number × 10^{-4}	[^{3}H]Thy incorporated per cell (dpm/cell) (%)[b]
None	7814 ± 275	9.6 ± 0.6	0.08 ± 0.01 (100)
EGCG (20 μM)	11564 ± 310	8.3 ± 0.5	0.14 ± 0.01 (175)
MTX (1 μM)	13140 ± 500	7.3 ± 0.6	0.18 ± 0.01 (225)

[a]Results are the mean ± SD of five determinations from three independent experiments.
[b]Percentage of [^{3}H]Thy incorporation respect to untreated control.



Scheme 1. Navarro-Perán et al.

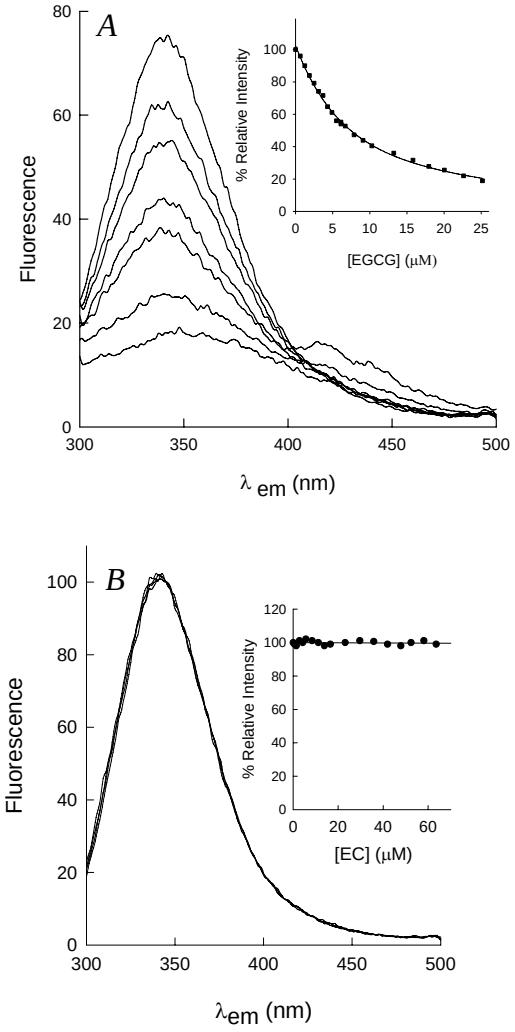


Fig. 1. Navarro-Perán et al.

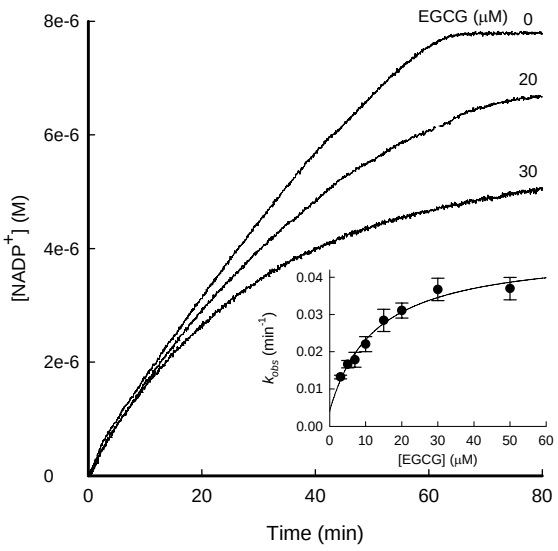


Fig. 2. Navarro-Perán et al.

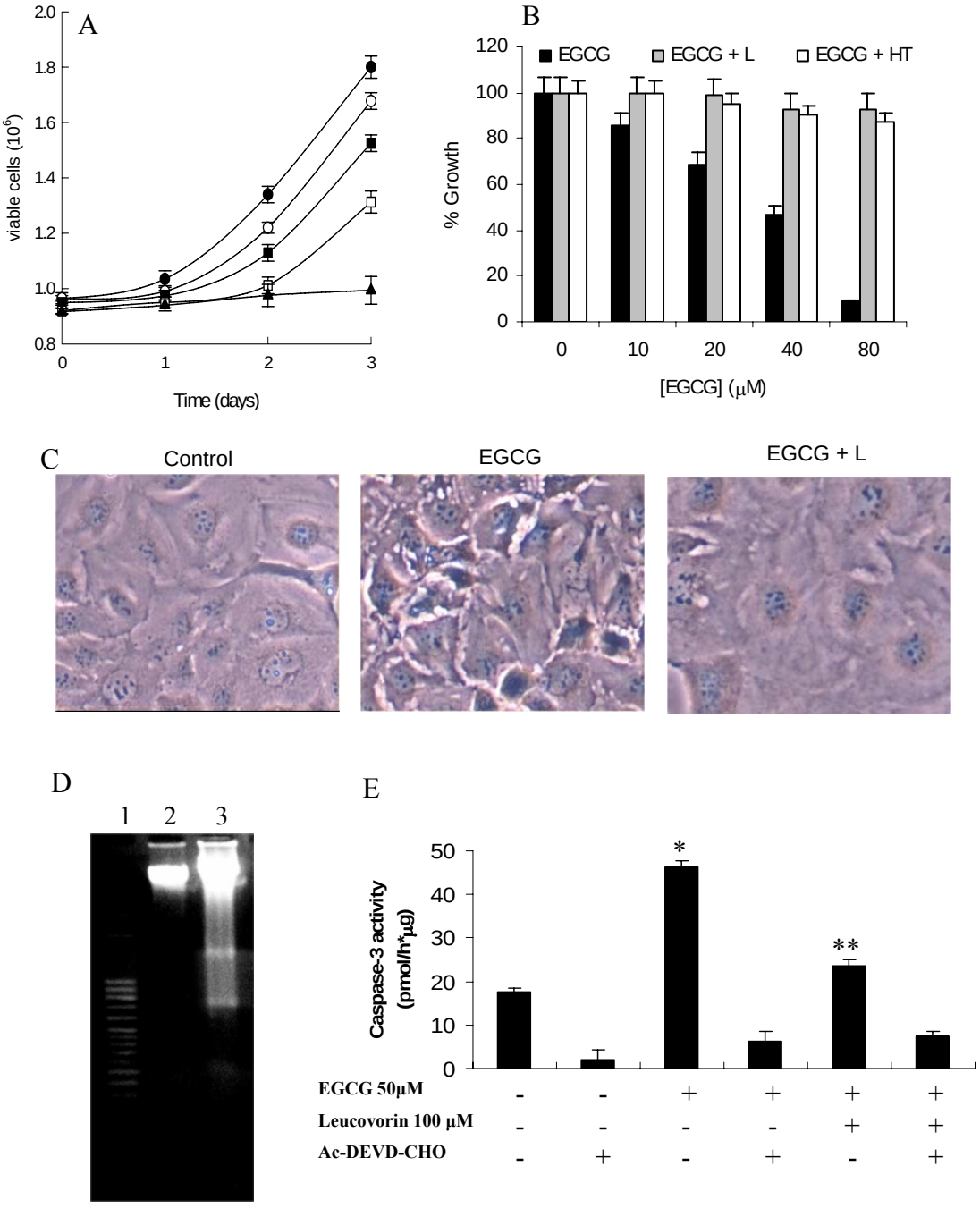
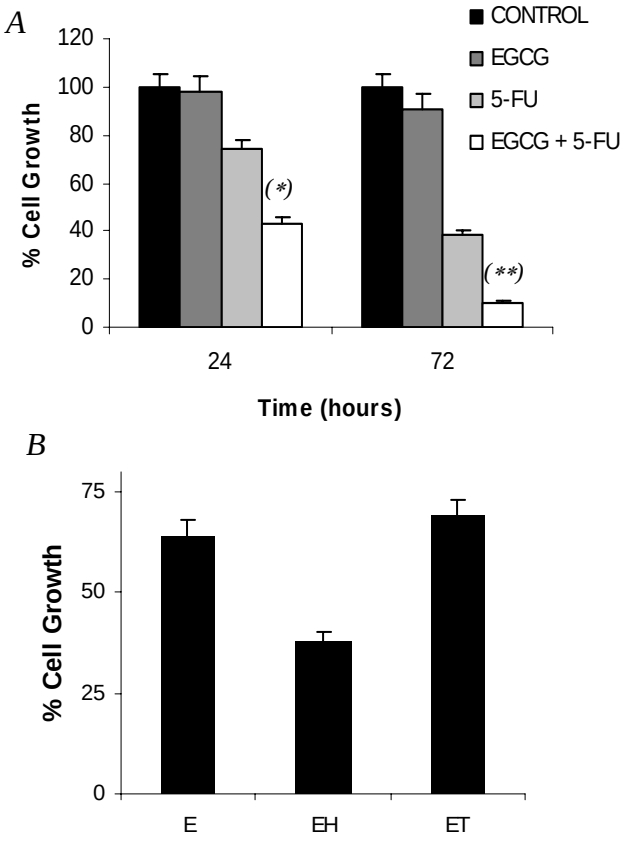


Fig. 3. Navarro-Perán et al.



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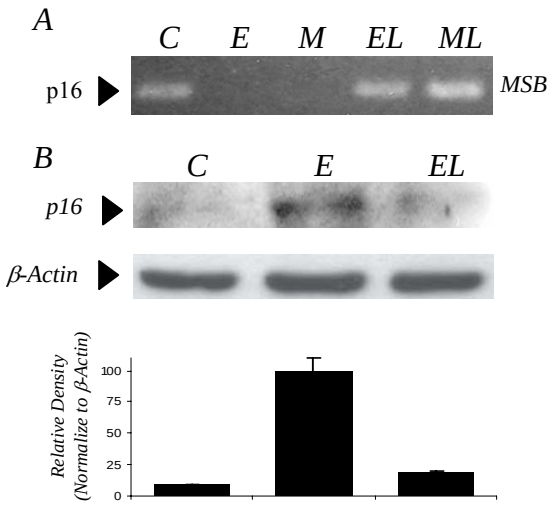

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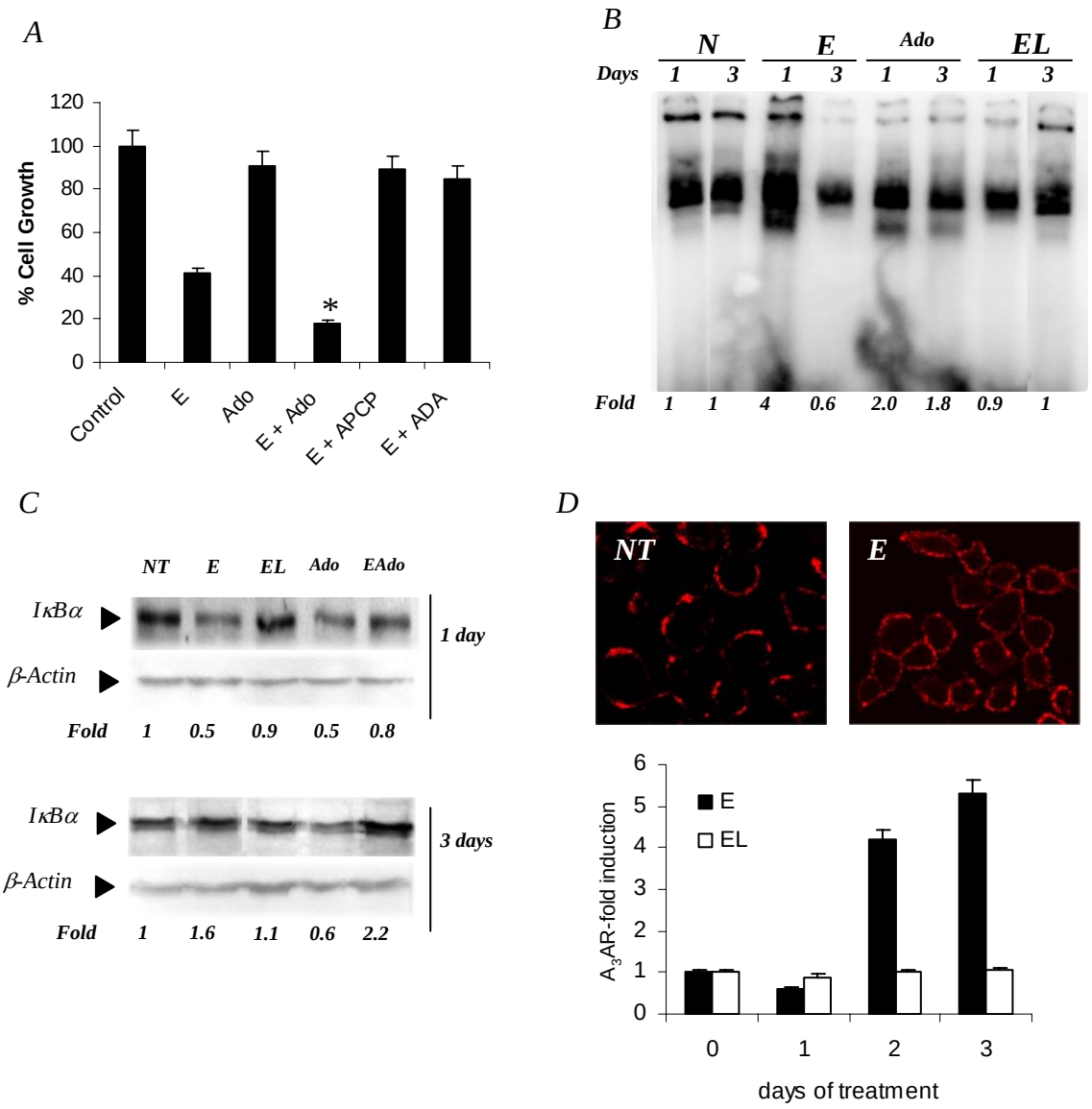

Figure 6. Navarro-Perán et al.