Epicatechin gallate induces cell death via p53 activation and stimulation of p38 and JNK in human colon cancer SW480 cells

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Abstract

The tea flavonoid epicatechin gallate (ECG) exhibits a wide range of biological activities. In this study, the *in vitro* anticancer effects of ECG on SW480 colon cancer cell line was investigated by analyzing the cell cycle, apoptosis, key proteins involved in cellular survival/proliferation, namely AKT/PI3K and MAPKs, and the role of p53 in these processes. ECG induced cell cycle arrest at the G0/G1-S phase border associated with the stimulation of p21, p-p53 and p53 and the suppression of cyclins D1 and B1. Exposure of SW480 cells to ECG also led to apoptosis as determined by time-dependent changes in caspase-3 activity, MAPKs (ERK, p38 and JNK), p21 and p53 activation, and AKT inhibition. The presence of pifithrin, an inhibitor of p53 function, blocked ECG-induced apoptosis as was manifested by restored cell viability and caspase-3 activity to control values, and reestablished the balance among Bcl-2 anti- and pro-apoptotic protein levels. Interestingly, ECG also inhibited p53 protein and RNA degradation, contributing to the stabilization of p53. In addition, JNK and p38 have been identified as necessary for ECG-induced apoptosis, upon activation by p53. The results suggest that the activation of the p53-p38/JNK cascade is required for ECG-induced cell death in SW480 cells.

Keywords: Cell death, colon cancer, epicatechin gallate, p53.

1. Introduction

Colorectal cancer (CRC) is a common malignancy ranking third in frequency on the world and constitutes the most common malignancy in developed countries (1, 2). It has been described that one-third of human carcinoma might be associated with dietary habits and lifestyle (1, 2). Thus, one promising approach to reduce the incidence and improve the prognosis of CRC may be chemoprevention/chemotherapy through diet.

The development of CRC involves various genetic and molecular changes in cell proliferation, survival and differentiation, resistance to apoptosis, metastasis, and tumour angiogenesis (3). In this line, the misregulation of the cell cycle by altering the tight association of cyclins and cyclin-dependent kinases (CDKs) or cyclin-dependent kinases inhibitors (CDKIs) could lead to the stimulation or inhibition of cell growth. Similarly, the protein kinase B (AKT)/phosphatidylinositol-3-kinase (PI3K) pathway is involved in cell survival/proliferation (3-5), and mitogen-activated protein kinases (MAPKs), especially c-jun amino-terminal kinase (JNK) and p38, seem to be related to stressful situations or apoptosis induction (6). In addition, as most human cancers, colon cancer exhibits mutations of the *p53* gene and altered protein function, which promotes an inappropriate cell survival, allowing a continued proliferation and evolution of the damaged cells (3, 7). In fact, mutations of *p53* are considered to be an early step in the development of CRC and could be correlated to aggressive, chemoresistant tumours, especially in patients with CRC (3, 7). *P53* plays a central role in the response to a wide range of cellular stresses, leading to cell cycle arrest, cell senescence or apoptosis (7-9), and it has been reported that *p53* modulates the intrinsic mitochondrial apoptotic pathway and can be activated by more than one kinase (7).

(-)-Epicatechin gallate (ECG), a major tea catechin (5), seems to exert health benefits, including the prevention of cancer (10-12). Different studies have shown that ECG is a potent chemopreventive agent able to interfere with the oxidative/antioxidative potential of colonic

cells (13), to induce inhibition of cell growth, cell cycle arrest, and to trigger apoptosis in different cancer cell lines such as cells from human oral cavity (11), lung and colon (14-17), prostate (18), and in head and neck squamous cancer cells (19). However, the precise mechanism for the chemopreventive/chemotherapeutic activities of ECG remains largely unknown.

In this study, SW480 cells, an invasive cell line commonly used in metastatic studies (20), which exhibit a mutated *p53* gene that retains its ability to induce DNA repair, cell cycle arrest and apoptosis have been selected to determine the efficacy of ECG against colon cancer cells (21, 22). Therefore, SW480 cells represent one of the best characterized colorectal cancer cell lines, and constitute an interesting and widely used *in vitro* model for the study of this neoplasia (20, 21).

In previous studies, we have shown that ECG has anticarcinogenic potential through the induction of apoptosis in SW480 cells (16). The present study evaluates the underlying mechanisms of the *in vitro* anticancer effects of ECG on SW480 cells and demonstrates that ECG induces cell death associated to the alteration of the cell cycle, the inhibition of AKT, the stimulation of the MAPKs (ERK, JNK and p38), and the activation of p53. In addition, the relevance of p53 in cell death has been evaluated and it is shown that ECG stabilizes p53 and modulates p53 through JNK and p38.

2. Materials and methods

Materials and chemicals

ECG was obtained from Extrasynthese (Genay, France). Gentamicin, penicillin G, streptomycin, SP600125, SB203580, PD98059, cycloheximide (CHX), actinomycin D (ActD), crystal violet and propidium iodide were purchased from Sigma Chemicals (Madrid, Spain). RNase A was obtained from Roche Molecular Biochemicals (Barcelona, Spain). Anti-cyclin B1, anti-phospho-Ser15-p53, anti-AKT and anti-phospho-Ser473-AKT, as well as anti-ERK1/2 and anti-phospho-ERK1/2 recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK and anti-phospho-Thr183/Tyr185-JNK, anti-phospho-p38 recognizing phosphorylated Thr180/Tyr182, p21 and anti- β -actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-cyclin D1 (sc-718), anti-Bclx (sc-634), anti-Bad (sc-943), anti-phospho-Ser112-Bad (sc-7998) and anti-p38 α (sc-535) were purchased from Santa Cruz Biotechnology (Qimigen, Madrid, Spain). Caspase-3 substrate (Ac-DEVD-AMC) and anti-p53 were from BD Pharmingen (Madrid, Spain), and pifithrin (PF) was obtained from Calbiochem (Merck, Madrid, Spain). Materials and chemicals for electrophoresis and the Bradford reagent were obtained from BioRad (Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and foetal bovine serum (FBS) from Lonza (Madrid, Spain).

Cell cultures and ECG treatments

Human SW480 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) F-12 medium, supplemented with 10% foetal bovine serum (FBS) and 50 mg/L of each antibiotic (gentamicin, penicillin and streptomycin). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. SW480 cells were a gift from Prof. Alberto Muñoz (Instituto de Investigaciones Biomedicas “Alberto Sols”, CSIC, Madrid, Spain).

Cells were changed to serum-free medium 24 h before the assay in order to avoid the influence of the growth factors contained in the FBS on the results. To study the cellular effect of ECG, different concentrations (10-50 μ M), diluted in serum-free culture medium, were added to the cell plates for 24 h. At the end of the incubation period, cell cultures were processed according to the assay (see below).

To study the time-course effects of ECG, cells were treated with 30 μ M ECG and then harvested at different times (0, 2, 4, 8, 16 and 24 h). In the experiments with ECG and ActD or CHX, cells were preincubated with 10 μ g/mL ActD (1 h) or 20 μ g/mL CHX (2 h) prior to 30 μ M ECG for 0, 2, 4, 8, 16 or 24 h. In panels B and D (Figure 4), right sets of columns, “0” column corresponds to cells only treated with 30 μ M ECG for 24 h.

Inhibition of p53 was performed by preincubating the cells for 16 h with 10 μ M pifithrin and then the treatment with 30 μ M ECG for 8 h or 24 h. In the case of inhibition of ERK-MAPK, JNK-MAPK or p38-MAPK was performed by pretreatment for 1 h with 50 μ M PD98059 (a specific inhibitor of ERK), 40 μ M SP600125 (a specific inhibitor of JNK), or 10 μ M SB203580 (a specific inhibitor of p38), respectively and later incubated with 30 μ M ECG for 8 or 24 h.

Cell viability assay

Cellular damage was evaluated by crystal violet assay (23). Cells were seeded (2×10^5 cells per well) in 24-well plates, grown for 20 h with the different treatments and then incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1% sodium dodecylsulfate added. The absorbance of each well was measured at 570 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Flow cytometry analysis

The distribution of cells in the sub-G1 (hypodiploid peak), G1, G2-M, and S phases of the cell cycle was determined by flow cytometric analysis of DNA content after the corresponding treatment. DNA was determined after labelling the cells with propidium iodide (24). Briefly, cell suspensions $[(0.5-1) \times 10^5 \text{ cells}]$ were prepared by trypsinization and washing twice with PBS, followed by centrifugation at 200 g. Cells were fixed with 70% ethanol at 4 °C and resuspended in PBS containing 0.25 mg/mL of RNase A. The suspension was incubated for 30 min at 37°C, and then the cells were labelled with propidium iodide (50 µg/mL). The total DNA content was quantified by fluorescence using a Becton Dickinson (San José, CA) FACS flow cytometer. The resulting histogram was analysed using ModFit software.

Fluorometric analysis of caspase-3 activity

Caspase-3 activity was measured as previously described (25). Detached cells were collected by centrifugation at 250 g for 10 min at 4°C. Attached cells were scrapped off in PBS and pelleted by centrifugation at 250 g for 10 min. Cells were pooled and lysed at 4°C in a buffer containing 5 mM Tris (pH 8), 20 mM EDTA and 0.5% Triton-X100. Later, lysates were clarified by centrifugation at 13000 g for 10 min. The reaction mixture contained 20 mM HEPES (pH 7), 10% glycerol, 2 mM dithiothreitol (DTT), 30 µg protein/condition and 20 µM Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was determined by measuring fluorescence in a microplate reader at an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Preparation of total cell lysates for Western blotting

To detect cyclin D1, cyclin B1, p21, Bcl-x, Bad, phospho-Bad, p53, phospho-p53, AKT, phospho-AKT, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38 and phospho-p38 cells were lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2

mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 200 mM β -glycerolphosphate, 0.1 mM Na_3VO_4 , 2 $\mu\text{g/mL}$ leupeptin and 1 mM phenylmethylsulfonyl fluoride. The supernatants were collected, assayed for protein concentration by using the Bio-Rad (Madrid, Spain) protein assay kit according to the manufacture's specifications, aliquoted and stored at -80°C until used for Western blot analyses.

Western blot analysis

Equal amounts of proteins (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Millipore, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit immunoglobulin (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Equal loading of Western blot was ensured by β -actin and bands were quantified using a scanner and accompanying software.

Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P < 0.05$ was considered significant. A SPSS version 19.0 program has been used.

3. Results

ECG affects cell cycle progression in SW480 cells.

To determine the potential effects of ECG on cell cycle, SW480 cells were exposed to a range of concentrations (10-50 μ M) of ECG for 24 h and then harvested to evaluate the cell cycle distribution by flow cytometry.

Treatment with ECG induced a significant decrease of cells in G2/M phase at concentrations higher than 20 μ M.. In addition to the G0/G1 arrest, a significant proportion of cells entered in apoptosis indicated by the appearance of a sub-G1 peak (Figure 1A).

The effects of ECG on cyclin D1, cyclin B1, CDKI p21, p-p53 and p53 were assayed by Western immunoblotting. As shown in Figures 1B and 1C, ECG-treated cells exhibited decreased protein levels of cyclin B1 and cyclin D1 at 30 μ M ECG.

P53 is one of the most important molecules in the regulation of cell growth and apoptosis (7-9). P53 becomes activated through phosphorylation and stabilization in response to various types of stress, and consequently could arrest cell cycle predominantly in G0/G1 phase via induction of the CDKI p21 (8). Thus, 20-50 μ M ECG caused an increase in the levels of p21, p-p53 and p53 after 24 h of treatment as compared to untreated SW480 cells. All these results could indicate a cell cycle arrest at the G0/G1-S phase border and induction of apoptosis (Figure 1).

Since 30 μ M ECG was the lowest concentration that altered the cell cycle distribution and levels of cell cycle-related proteins in SW480 cells, this was the concentration selected for studying the effects through time on cell cycle-related proteins, apoptosis and survival/proliferation pathways.

ECG alters cell cycle and survival/proliferation through time in SW480 cells.

In view of the alterations observed for 30 μ M ECG on cell cycle, the potential apoptotic effect of this flavanol was tested in SW480 cells by a time-course (0-24 h) evaluation of caspase-3 activity, which is a crucial effector in the terminal or execution phase of the apoptotic pathway,

and key proteins of survival/proliferation pathways. As shown in Figure 2A, ECG (30 μ M) increased caspase-3 activity at 8-24 h, and caused a rapid (4 h onwards) and marked increase in protein levels of p21, p-p53 and p-53 (Figures 2B and 2C).

PI3K/AKT and MAPKs play an important role in cell survival, and can be modulated by polyphenols (4, 12). Thus, it was investigated whether treatment with ECG was able to regulate key proteins of these intracellular signalling cascades in SW480 cells. ECG induced an early decrease in AKT phosphorylated levels (4-24 h) and a strong activation of ERK (2-24 h), whereas an increase in the phosphorylated levels of p38 and JNK was observed from 8 h up to 24 h of incubation (Figures 2B and 2D). The protein expression levels of total AKT, ERK, JNK and p38 were not modified by ECG treatment. Therefore, the results suggested that 30 μ M ECG triggers apoptosis and that modulation of p21, p-p53 and p-53, as well as activation of ERK, p38 and JNK and inhibition of AKT might contribute to the apoptotic effect.

ECG-induced apoptosis is dependent on p53 activation.

Induction of apoptosis by chemopreventive/chemotherapeutic agents is often mediated through p53-dependent mechanisms (7, 9), and the reactivation of p53 has been suggested to be an effective strategy for cancer therapy in wild-type p53-retained tumour cells (7, 9). Thus, to evaluate the role of the activation of p53 in ECG-induced apoptosis, a selective inhibitor of this protein (pifithrin) was used and the results on cell viability and apoptosis were determined.

Treatment of SW480 cells with pifithrin followed by the addition of 30 μ M ECG returned cell viability and caspase-3 activity to similar values to those of control cells (Figures 3A and 3B).

An imbalance in the expression of anti- and pro-apoptotic proteins is one of the major mechanisms underlying the ultimate fate of cells in the survival/apoptotic process (25), and p53 could also contribute to promote mitochondrial permeabilisation, and thereby the induction of apoptosis (7). Thus, the possible modulation by ECG of the expression of anti- or pro-apoptotic

members of this family of proteins (Bcl-x and Bad, respectively) in the presence or absence of pifithrin was studied in the same conditions. Consistent with all the above, treatment of SW480 cells with 30 μ M ECG for 24 h altered the balance between anti- and pro-apoptotic Bcl-2 family members.

Bcl-x_L is a caspase substrate, and the product of Bcl-x_L cleavage, Bcl-x_S, has a proapoptotic function. This proteolytic fragment (Bcl-x_S) was detected when the cells were incubated for 24 h with ECG, and the Bcl-x_L/Bcl-x_S ratio was recovered to control values in the presence of pifithrin (Figures 3C and 3D). ECG also induced an enhancement of Bad levels and p21, whereas treatment with the p53 selective inhibitor showed values lower than those of control cells (Figures 3C and 3D). On the contrary, p-Bad levels decreased in the presence of ECG, although p-Bad values were enhanced in the presence of ECG+PF (Figures 3C and 3D). Taken all together, it could be suggested that the increase of p53 is required for ECG-induced apoptosis in SW480 cells.

ECG stabilizes p53.

To test whether the stability of p53 protein levels was affected by ECG, SW480 cells were treated with 20 μ g/mL CHX, a protein synthesis inhibitor, for 2 h prior to 30 μ M ECG incubation and cells were harvested at different incubation times (0, 2, 4, 8, 16 or 24 h).

Treatment with ECG seemed to delay the degradation of p53, as its protein levels were lower in CHX-treated cells than in ECG+CHX-treated cells, i.e. p53 protein values were higher at all incubation times in the presence of the flavanol (Figures 4A and 4B).

In order to clarify whether ECG-induced p53 protein also depended upon transcriptional regulation, cells were pretreated with 10 μ g/mL ActD, a transcriptional inhibitor, for 1 h prior to 30 μ M ECG incubation (0-24 h). As happened for CHX, ActD decreased p53 levels through time and showed values below control cells at the longest incubation times (16 and 24 h, Figures 4C

and 4D). However, in the presence of 30 μ M ECG, p53 protein levels were enhanced at 2-8 h to later return to control values (Figures 4C and 4D). All these results indicate that ECG stabilizes p53 at both protein and mRNA levels.

Activation of MAPKs by ECG is mediated by p53.

MAPKs (ERK, JNK and p38) can be activated by ECG in different cell types (16, 26, 27), although the mechanism of activation remains unclear and the precise connection between p53 and MAPKs upon ECG treatment has not previously been studied. To analyse the role of p53 on the activation of MAPKs, a selective inhibitor of p53 was used and the effect on the modulation of MAPKs was evaluated by Western immunoblot.

ECG increased p-ERK, p-JNK and p-p38 levels in SW480 in a time dependent manner, showing all of them a strong activation after 8 h of treatment (Figure 2). Thus, this was the selected time to perform the specific experiments; SW480 cells were pretreated with pifithrin for 16 h, then incubated for 8 h with 30 μ M ECG and later, protein specific immunoblots were then performed. Treatment with ECG induced the phosphorylation of ERK, JNK and p38. However, the incubation of cells with the p53 inhibitor completely abolished the ECG-enhanced phosphorylated levels of JNK and p38, whereas p-ERK values remained elevated (Figures 5A and 5B). Likewise, there was no difference in the total protein levels of MAPKs (ERK, JNK and p38). These results suggest that p53 modulates the activation of JNK- and p38-MAPKs in SW480 cells.

Activation of MAPKs by ECG is required for p53 stimulation and apoptosis induction.

To determine the relevance of the MAPKs activation in ECG-induced cell death and the involvement of p53 in these processes in SW480 cells, the effect of MAPKs inhibition was analysed. As shown in Figure 6A, pretreatment of cells with SP600125 completely abolished ECG-induced cell death, while SB203580 and PD98059 showed a slight although yet significant

effect in diminishing the cell damage induced by the flavanol. In addition, the inhibitors of JNK and p38, SP600125 and SB203580, respectively, partly abrogated the ECG-induced apoptosis as shown by a lower caspase-3 activity in comparison with ECG-treated cells, whereas the ERK inhibitor PD98059 showed a modest effect on ECG-induced caspase-3 activity (Figure 6B). In this line, pretreatment of SW480 cells with SP600125 and SB203580 decreased the amount of p-p53 and total p53 protein after 8 h of incubation with 30 μ M ECG, whereas both levels of proteins (p-p53 and p53) remained enhanced after the blockage of ERK by PD98059 pretreatment (Figures 6C and 6D). These results indicate that JNK and p38 are involved in ECG-induced cell death, whereas ERK seemed to play a minor role.

4. Discussion

CRCs are more resistant to chemotherapy as compared to other cancer types (2, 3). It is, therefore, important to devise strategies making the chemoresistant tumour cells more sensitive to drugs. Different epidemiological studies have shown that a high consumption of fruits and vegetables, which are rich in phenolic compounds, reduce the risk of CRC (2). The discovery of effective anticancer plant-derived natural products plays an important role in cancer chemotherapy (4, 5, 12). In this line, induction of apoptosis and cell growth inhibition in different cancer cell lines by ECG has been demonstrated (11, 16, 17, 19, 28), although the precise mechanism has not been fully characterized.

In the current study, SW480 cells were used to provide *in vitro* evidence that ECG could induce G0/G1-S phase arrest of cell cycle and apoptotic cell death. We have demonstrated that ECG contributes to stabilize p53 and that the p53 tumour suppressor is essential to induce apoptosis. Moreover, MAPKs (JNK and p38) are activated through p53 and they also regulate p-p53 and total p53 levels, since MAPKs inhibition interferes with this process and with the induction of the apoptotic cell death.

A major portion of ingested polyphenols survive digestion in the upper gastrointestinal tract and reach the colon, where they could be subjected to fermentation by colonic microflora (10). This observation has been confirmed in human ileostomy patients (29). Therefore, despite the low serum bioavailability of flavonoids (10), due to the high catechin content of green tea, cocoa and other well accepted foodstuffs, colonic cells could be exposed to flavanol levels similar or higher to those tested in the present study. In this line, it has been reported that the concentration of ECG in the lumen may range between 75 and 300 μM after having a cup of tea and that ECG is extensively taken up by colonic cells (30).

Cell growth is controlled by several genetically defined checkpoints that ensure its coordinated progression through the different stages of the cell cycle and monitor DNA integrity (8). The

analysis of the cell cycle after treatment of SW480 cells with different concentrations of ECG showed that this flavanol induces cell cycle arrest in G0/G1. Previous studies have indicated that D-type cyclins play an important role in tumour development and are overexpressed in various types of cancers, including colon cancer (31). In agreement with the present results, ECG induced cell cycle arrest in G1 phase via down-regulation of cyclin D1 in SCC7 cells (19) and inhibited growth of pancreatic and prostatic cancer cells (18). In addition, cell cycle arrest in G0/G1 phase diminished cyclin D1 levels and a selective decrease of cyclin B1 have already been reported in cell culture after the incubation with anticarcinogenic drugs (32). Cell cycle arrest induced by ECG was also accompanied with a significant increase in p21 levels. Accordingly, other natural compounds such as the diterpene triptolide (33), berberine (23) and a grape seed extract (34) induced cell cycle arrest and an upregulation of p21 in SW480 cells, suggesting that p21 was associated to the regulation of cell turnover.

During chemotherapy, accumulation of cells in G0/G1 is often mediated by activation of p53 causing, besides cell cycle arrest predominantly at G1 phase, induction of apoptosis (19, 28) in agreement with the results of the present work. Correspondingly, apoptosis induction was correlated with the increased levels of p-p53, as previously shown for resveratrol in colon cancer cells (35) and baicalin in breast cancer cells (36). It has also been reported that ECG suppresses abnormal cell growth from B[a]P-induced lung carcinogenesis by inducing p53 and its downstream targets such as p21-CDKI, along with cyclin D1 at different time points (15). In this regard, ECG-induced cell death appeared to be dependent on p53 in lung carcinogenesis and in SCC7 and NCI-H460 cell lines (14, 15, 19). Similarly, a p53-dependent apoptosis was induced by epigallocatechin gallate (EGCG) in different colon (37) and in A549 lung cancer cells (38). EGCG, grape seed extract, quercetin and resveratrol also led to decreased levels of cyclin D1, increased p21, and induction of p53, which played a critical role in the induction of apoptosis in colon cancer cells (28, 34, 35, 37, 39, 40). Although p53 appears to be a key regulator of p21,

abundant evidence indicates that p21 can also be induced in a p53-independent manner. In this regard, Kaur et al. (34) have reported that a grape seed extract stimulated p21 to arrest SW480 cell cycle, and this effect was independent of p53, showing that the response was comparable in colon cancer cells harbouring mutated and wild-type p53.

The downstream targets of p53 to execute apoptosis are wide and vary with the cell type and stimuli (7). In this study, the down-regulation of Bcl-x_L and p-Bad, and up-regulation of Bcl-x_S, Bad and p21 were observed in ECG-treated SW480 cells, which were abrogated by the blockage of p53. As previously mentioned, ECG-induced apoptosis has been reported in p53 wild-type colon cancer cells (27, 41). ECG also induced caspase-3 activity and DNA fragmentation, indicative of apoptosis, in human oral cancer cells but not in normal fibroblasts (11). Moreover, ECG induced apoptosis by increasing the expression of p53 and the pro-apoptotic Bax and reducing the levels of anti-apoptotic Bcl-2 and Bcl-x_L in lung cancer cells (14, 15). In this line, EGCG, baicalin, quercetin, lupulone and curcumin also enhanced p53 values and the ratio of Bax/Bcl-2 in favour of apoptosis (36, 37, 40, 42). Similarly, ECG also induced cell growth inhibition and up-regulated two apoptosis-related genes (*GADD153* and *p21*) by directly interacting with the nucleic acids in human cancer cells (43). However, it should be mentioned that ECG is able to induce growth inhibition and apoptosis in p53 wild-type colon cancer HCT116 cells in a p53-independent manner (41). Additionally, Bad, which is constitutively phosphorylated at Ser112 and to a lesser degree, Ser136 in SW480 cells (44), also seemed to play a role in the induction of apoptosis. In this work, it is demonstrated that ECG inhibited phosphorylation of Bad and increased unphosphorylated Bad levels, and p53 seems to be involved in this process. Therefore, a role for Bad and p-Bad in the modulation of apoptosis is demonstrated in SW480 cells, as previously shown (44).

The degradation of p53 is one of the main alterations for the activation of this tumour suppressor. Thus, stabilization of the p53 protein has been suggested to be an effective strategy

for cancer therapy (9). A similar effect has already been reported for other polyphenols and anticarcinogenic drugs (9, 40). In this regard, the flavonoid quercetin stabilized p53 at post-transcriptional level by inhibiting p53 ubiquitination and protein turnover at the post-translation level in HepG2 cells (40). This might contribute to ensure p53-dependent actions such as cell cycle arrest and apoptosis, but further studies are needed to elucidate how p53 is stabilized by ECG in SW480 cells.

MAPKs and PI3K/AKT pathways play an important role in regulating chemically induced apoptosis (4) and up-regulation of AKT has been considered a critical factor in the progression of CRC (3). Thus, decreased levels of p-AKT (Thr308) and/or p-AKT (Ser473) induced by natural compounds have been accompanied with apoptosis induction and/or antiproliferative effects in colon cancer cells (33, 45). Among MAPKs, ERK is involved in regulating cell growth and differentiation (3, 4), although diverse studies have suggested that the role of ERK in determining the fate of cells (survival or death) is cell type specific (6). In contrast to ERK, JNK and p38 MAPK are activated by stress inducing signals such as oxidative stress, environmental stress, and pro-apoptotic treatments (6). ECG induced an early enhancement of p-ERK followed by an increase in p-JNK and p-p38, as well as in caspase-3 activity together with a diminution of p-AKT levels in SW480 cells. In line with this, it has been reported that ECG induced the activation of ERK, JNK and p38 in HCT116 colorectal cancer cells leading to the inhibition of the cell growth and induction of apoptosis (27). In addition, ECG and EGCG caused a sustained activation of ERK and p38, and all three MAPK, respectively in HepG2 cells, and these stimulations were associated to the apoptotic cell death (26). Similarly, other natural compounds enhanced p-ERK, p-p38 levels and decreased p-AKT values contributing to the induction of apoptosis in different colon carcinoma cells (16, 46, 47).

MAPKs seem to be involved in p53-regulated apoptosis (9, 21), although the role of MAPKs is mainly context-dependent and is influenced by cell type and the nature of stress (6, 16, 21). The

present study shows that the protein levels of p-JNK and p-p38 were up-regulated by p53 in ECG-treated SW480 cells, whereas p-ERK values seemed not to be modulated by p53. Similarly, a role for p38 and JNK has been reported in the modulation of the p53-mediated apoptosis in colon cancer cells by different natural compounds, such as lupulone and maslinic acid (42, 48). In this line, both ERK and p38 mediated resveratrol-induced activation of p53 in mouse epidermal cells (39), being JNK one of the key regulatory steps in resveratrol-induced p53 activation and p53-mediated apoptosis (49). Moreover, in the present study the specific inhibition of MAPKs was able to counteract the apoptotic effect induced by ECG in the SW480 cell line. In agreement with our results, berberin and EGCG induced apoptosis through an activation of JNK and p38 pathways in colon cancer cells (27, 50) and hepatoma cells (26), and the blockage of p38 prevented the lupulone-induced apoptosis in SW620 colon carcinoma cells (42). In addition, the inhibition of p38, JNK and ERK impaired the resveratrol-induced programmed cell death in JB6 mouse epidermal cells (39, 49). Therefore it can be suggested that in our study MAPKs (ERK, p38 and JNK) were involved in ECG-induced apoptosis in SW480 cells, and that p38 and JNK played a role in ECG-induced p53 activation.

In summary, our study shows for the first time that realistic doses of ECG inhibited the growth and induced apoptosis in colon SW480 cells associated to down-regulation of the PI3K/AKT pathway and the stimulation of MAPKs signalling and p53 (Figure 7). Our results indicate that ECG induced p53-mediated apoptosis and stabilized p53 mRNA and protein in SW480 cells. In addition, ECG activated the MAPK pathways via p53. Thus, stimulation of p38 and JNK contribute to increase p53 levels, which could lead to the amplification of the p53 signal. These findings will provide new insights into the complex mechanisms related to the cancer chemopreventive/chemotherapeutic properties of plant flavonoids.

Abbreviations used

ActD, actinomycin D; AKT/PKB, protein kinase B; CDK, cyclin-dependent kinases; CDKI, cyclin-dependent kinases inhibitor; CHX, cycloheximide; CRC, colorectal cancer; DTT, dithiothreitol; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; ERK, extracellular regulated kinase; FBS, foetal bovine serum; MAPK, mitogen-activated protein kinases; JNK, c-jun amino-terminal kinase; PI3K, phosphatidylinositol-3-kinase.

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5. References

1. Chan AT and Giovannucci EL: Primary prevention of colorectal cancer. *Gastroenterol* **138**, 2029-2043, 2010.
2. Half E and Arber N: Colon cancer: preventive agents and the present status of chemoprevention. *Expert Opin Pharmacother* **10**, 211-219, 2009.
3. Pan M-H, Lai C-S, Wu J-C and Ho C-T: Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. *Mol Nutr Food Res* **55**, 32-45, 2011.
4. Ramos S: Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol Nutr Food Res* **52**, 507-526, 2008.
5. Yang CS and Xin Wang X: Green tea and cancer prevention. *Nutr Cancer* **67**, 931-937, 2010.
6. Zhou Y, Wang Q, Evers BM and Chung DH: Signal transduction pathways involved in oxidative stress-induced intestinal epithelial cell apoptosis. *Pediatr Res* **58**, 1192-1198, 2005.
7. Fridman JS and Lowe SW: Control of apoptosis by p53. *Oncogene* **22**, 9030-9040, 2003.
8. Damia G and Broggini M: Cell cycle checkpoint proteins and cellular response to treatment by anticancer agents. *Cell Cycle* **3**, 46-50, 2004.
9. Fuster JJ, Sanz-González SM, Moll UM and Andrés V: Classic and novel roles of p53: prospects for anticancer therapy. *Trends Mol Med* **13**, 192-199, 2007.
10. Aron PM and Kennedy JA: Flavan-3-ols: Nature, occurrence and biological activity. *Mol Nutr Food Res* **52**, 79-104, 2008.

11. Babich H, Krupka M, Nissim H and Zuckerbraun H: Differential in vitro cytotoxicity of (-)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. *Toxicol in Vitro* **19**, 231-242, 2005.
12. Giovannini C and Masella R: Role of polyphenols in cell death control. *Nutr Neurosci* **15**, 134-149, 2012.
13. Rodríguez-Ramiro I, Martin MA, Ramos S, Bravo L and Goya L: Comparative effects of dietary flavanols on antioxidant defences and their response to oxidant induced stress on Caco2 cells. *Eur J Nutr* **50**, 313-322, 2011.
14. Ganguly C, Saha P, Panda CK and Das S: Inhibition of growth, induction of apoptosis and alteration of gene expression by tea polyphenols in the highly metastatic human lung cancer cell line NCI-H4601. *Asian Pacific J Cancer Prev* **6**, 326-331, 2006.
15. Manna S, Mukherjee S, Roy A, Das S and Panda CK: Tea polyphenols can restrict benzo[a]pyrene-induced lung carcinogenesis by altered expression of p53-associated genes and H-ras, c-myc and cyclin D1. *J Nutr Biochem* **20**, 337-349, 2009.
16. Ramos S, Rodriguez-Ramiro I, Martin MA, Goya L and Bravo L: Dietary flavanols exert different effects on antioxidant defenses and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. *Toxicol in Vitro* **25**, 1771-1781, 2011.
17. Yang G, Liao J, Kim K, Yurkow E and Yang C: Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **19**, 611-616, 1998.
18. Lyn-Cook BD, Rogers T, Yan Y, Blann EB, Kadlubar FF, et al.: Chemopreventive effects of tea extracts and various components on human pancreatic and prostate tumor cells in vitro. *Nutr Cancer* **35**, 80-86, 1999.

19. Lim YC, Lee SH, Song MH, Yamaguchi K, Yoon JH, et al.: Growth inhibition and apoptosis by (-)-epicatechin gallate are mediated by cyclin D1 suppression in head and neck squamous carcinoma cells. *Eur J Cancer* **42**, 3260-3266, 2006.
20. Yoon WH, Lee SK, Song KS, Kim JS, Kim TD, et al.: The tumorigenic, invasive and metastatic potential of epithelial and round subpopulations of the SW480 human colon cancer cell line. *Mol Med Rep* **1**, 763-768, 2008.
21. Brantley-Finley C, Lyle CS, Du L, Goodwin ME, Hall T, et al.: The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by antitumor agents vinblastine, doxorubicin and etoposide. *Biochem Pharmacol* **66**, 459-469, 2003.
22. Tewari R, Sharma V, Koul N and Sen E: Involvement of miltefosine-mediated ERK activation in glioma cell apoptosis through Fas regulation. *J Neurochem* **107**, 616-627, 2008.
23. Murthy KNC, Jayaprakasha GK and Patil BS: The natural alkaloid berberine targets multiple pathways to induce cell death in cultured human colon cancer cells. *Eur J Pharmacol* **688**, 14-21, 2012.
24. Ramos S, Alia M, Bravo L and Goya L: Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). *J Agric Food Chem* **53**, 1271-1280, 2005.
25. Granado-Serrano A, Martín M, Bravo L, Goya L and Ramos S: Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-Kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). *J Nutr* **136**, 2715-2721, 2006.
26. Chen C, Yu R, Owuor E and Kong A: Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major

green tea polyphenol components during cell survival and death. *Arch Pharmacol Res* **23**, 605-612, 2000.

27. Inaba H, Nagaoka Y, Kushima Y, Kumagai A, Matsumoto Y, et al.: Comparative examination of anti-proliferative activities of (–)-epigallocatechin gallate and (–)-epigallocatechin against HCT116 colorectal carcinoma cells. *Biol Pharm Bull* **31**, 79-84, 2008.

28. Vanamala J, Reddivari L, Radhakrishnan S and Tarver C: Resveratrol suppresses IGF-1 induced human colon cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways. *BMC Cancer* **10**, 238, doi:10.1186/1471-2407-10-238, 2010.

29. Karle K, Kraus M, Scheppach W, Ackerman M, Ridder F, et al.: Studies on apple and blueberry fruits constituents: do polyphenols reach the colon after ingestion? *Mol Nutr Food Res* **50**, 418-423, 2006.

30. Vaidyanathan JB and Walle T: Cellular uptake and efflux of the tea flavonoid (–)-epicatechin-3-gallate in the human intestinal cell line caco-2. *J Pharmacol Exp Ther* **307**, 745-752, 2003.

31. Kwak YT, Radaideh SM, Ding L, Li R, Frenkel E, et al.: Cells lacking IKK{alpha} show nuclear cyclin D1 overexpression and a neoplastic phenotype: role of IKK{alpha} as a tumor suppressor. *Mol Cancer Res* **9**, 341-349, 2011.

32. Heffeter P, Jakupec MA, Körner W, Wild S, Graf von Keyserlingk N, et al.: Anticancer activity of the lanthanum compound [tris(1,10-phenanthroline)lanthanum(III)]trithiocyanate (KP772; FFC24) *Biochem Pharmacol* **71**, 426-440, 2006.

33. Liu J, Shen M, Yue Z, Yang Z, Wang M, et al.: Triptolide inhibits colon-rectal cancer cells proliferation by induction of G1 phase arrest through upregulation of p21. *Phytomedicine* **19**, 756-762, 2012.
34. Kaur M, Mandair R, Agarwal R and Agarwal C: Grape seed extract induces cell cycle arrest and apoptosis in human colon carcinoma cells. *Nutr Cancer* **60**, 2-11, 2008.
35. Radhakrishnan S, Reddivari L, Sclafani R, Das U and Vanamala J: Resveratrol potentiates grape seed extract induced human colon cancer cell apoptosis. *Front Biosci (Elite Ed)* **3**, 1509-1523, 2011.
36. Wang N, Tang LJ, Zhu GQ, Peng DY, Wang L, et al.: Apoptosis induced by baicalin involving up-regulation of p53 and bax in MCF-7 cells. *J Asian Nat Prod Res* **10**, 1129-1135, 2008.
37. Thakur VS, Ruhul-Amin AR, Paul RK, Gupta K, Hastak K, et al.: p53-dependent p21-mediated growth arrest pre-empts and protects HCT116 cells from PUMA-mediated apoptosis induced by EGCG. *Cancer Lett* **296**, 225-232, 2010.
38. Yamauchi R, Sasaki K and Yoshida K: Identification of epigallocatechin-3-gallate in green tea polyphenols as a potent inducer of p53-dependent apoptosis in the human lung cancer cell line A549. *Toxicol in Vitro* **23**, 834-839, 2009.
39. She QB, Bode AM, Ma WY, Chen NY and Dong Z: Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res* **61**, 1604-1610, 2001.
40. Tanigawa S, Fujii M and Hou DX: Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells. *Biosci Biotechnol Biochem* **72**, 797-804, 2008.

41. Baek SJ, Kim JS, Jackson FR, Eling TE, McEntee MF, et al.: Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis* **25**, 2425-2432, 2004.
42. Lamy V, Bousserouel S, Gossé F, Minker C, Lobstein A, et al.: Lupulone triggers p38 MAPK-controlled activation of p53 and of the TRAIL receptor apoptotic pathway in human colon cancer-derived metastatic cells. *Oncol Rep* 109-114, 2011.
43. Kuzuhara T, Tanabe A, Sei Y, Yamaguchi K, Suganuma M, et al.: Synergistic effects of multiple treatments, and both DNA and RNA direct bindings on, green tea catechins. *Mol Carcinogenesis* **46**, 640-645, 2007.
44. Popivanova BK, Li YY, Zheng H, Omura K, Fujii C, et al.: Proto-oncogene, Pim-3 with serine/threonine kinase activity, is aberrantly expressed in human colon cancer cells and can prevent Bad-mediated apoptosis. *Cancer Sci* **98**, 321-328, 2007.
45. Jia SS, Xi GP, Zhang M, Chen YB, Lei B, et al.: Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. *Oncol Rep* **29**, 349-354, 2013.
46. Hyun J-H, Kim S-C, Kang J-I, Kim M-K, Boo H-J, et al.: Apoptosis inducing activity of fucoidan in HCT-15 colon carcinoma cells. *Biol Pharm Bull* **32**, 1760-1764, 2009.
47. Auyeung KK, Mok NL, Wong CM and Ko JK: Astragalus saponins modulate mTOR and ERK signaling to promote apoptosis through the extrinsic pathway in HT-29 colon cancer cells. *Int J Mol Med* **26**, 341-349, 2010.
48. Reyes-Zurita FJ, Pachón-Peña G, Lizárraga D, Rufino-Palomares EE, Cascante M, et al.: The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer* **11**, 154, doi:10.1186/1471-2407-11-154, 2011.

49. She QB, Huang C, Zhang Y and Dong Z: Involvement of c-jun NH₂-terminal kinases in resveratrol-induced activation of p53 and apoptosis *Mol Carcinogenesis* **33**, 244-250, 2002.
50. Hsu WH, Hsieh YS, Kuo HC, Teng CY, Huang HI, et al.: Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL. *Arch Toxicol* **81**, 719-728, 2007.

Figure captions

Figure 1. Effect of ECG on cell cycle and levels of cyclin D1, cyclin B1, p21, phosphorylated and total p53 in SW480 cells after 24 h of treatment. (A) Cell distribution in cycle phases in SW480 cells after 24 h of treatment with different concentrations of ECG. Statistically significant differences *versus* control group at the same cell cycle phase ($P < 0.05$) are marked with distinct letters. (B) Blots of representative experiments. (C) Densitometric quantification of cyclin D1 and cyclin B1. (D) Percentage values of p21, p-p53 and p53 relative to controls. Values are expressed as a percentage relative to the control condition (means $\pm$ SD, $n = 5-6$). Equal loading of Western blots was ensured by β -actin. Means without a common letter differ ($P < 0.05$).

Figure 2. Time-course effect of 30 μ M ECG in SW480 cells. Caspase-3 activity and levels of p21, p-p53, p53, phosphorylated and total AKT, phosphorylated and total ERK, phosphorylated and total JNK, and phosphorylated and total p38 were assayed. (A) Caspase-3 activity (Units/mg protein). (B) Blots of representative experiments. (C) Densitometric quantification of p21, p-p53 and p53. (D) Percentage data of p-AKT/AKT, p-ERK/ERK, p-JNK/JNK and p-p38/p38 ratios relative to controls. Values are expressed as a percentage relative to the control condition and are means $\pm$ SD, $n = 5-7$. Equal loading of Western blots was ensured by β -actin. Means without a common letter differ ($P < 0.05$).

Figure 3. Effects of pifithrin (PF) pretreatment on ECG-induced apoptosis. Cells were incubated in the presence or absence of 10 μ M PF for 16 h and later treated with 30 μ M ECG for 24 h. Cell viability, caspase-3 activity and levels of Bcl-x_L/Bcl-x_S, p-Bad, Bad, and p21 were analysed in SW480 cells .. (A) Cell viability was determined by crystal violet and expressed as the relative

percentage of control cell staining. Data represent means $\pm$ SD of 8-10 different samples per condition. (B) Caspase-3 activity (Units/mg protein). (C) Blots of representative experiments. (D) Percentage values of Bcl-x_L/Bcl-x_S ratio, p-Bad and Bad and p21 levels relative to the control condition (means $\pm$ SD, n = 4-6). Equal loading of Western blots was ensured by β -actin. Different letters over bars indicate statistically significant differences ($P < 0.05$).

Figure 4. Effect of ECG on p53 stability. SW480 cells were preincubated with 20 μ g/mL CHX (2 h) or 10 μ g/mL ActD (1 h) prior to 30 μ M ECG incubation. Then, cells were collected at different times (0-24 h). (A) Blots of representative experiments. (B) Percentage data of p53 relative to controls. (C) Bands of representative experiments. (D) Densitometric quantification of p53. In panels B and D, right sets of columns, “0” column corresponds to cells only treated with 30 μ M ECG for 24 h. Values are expressed as a percentage relative to the untreated control condition and are means $\pm$ SD, n = 6-7. Equal loading of Western blots was ensured by β -actin. Means without a common letter differ ($P < 0.05$)

Figure 5. Effect of ECG and selective inhibitor pifithrin (PF) on levels of phosphorylated and total ERK, phosphorylated and total JNK, and phosphorylated and total p38. SW480 cells were incubated in the presence or absence of 10 μ M PF for 16 h and later with 30 μ M ECG for 8 h. (A) Blots of representative experiments. (B) Percentage data of p-ERK/ERK, p-JNK/JNK and p-p38/p38 ratios relative to the control condition (means $\pm$ SD, n = 5-6). Equal loading of Western blots was ensured by β -actin. Different letters over bars indicate statistically significant differences ($P < 0.05$).

Figure 6. Effects of ECG and selective inhibitors PD98059 (PD), SP600125 (SP) and SB203580 (SB) on cell death, caspase-3 activity and levels of phosphorylated and total p53 in SW480 cells.

Cells were incubated in the presence or absence of the inhibitors for 1 h and later with 30 μ M ECG for 24 h when cell viability was assayed or for 8 h to evaluate the caspase-3 activity and p-p53 and p53 levels. (A) Cell viability was expressed as percent of control (means $\pm$ SD, n= 8-10 different samples per condition). (B) Caspase-3 activity (Units/mg protein). (C) Blots of representative experiments. (D) Densitometric quantification of p-p53 and p53 levels Values are expressed as a percentage relative to the control condition and are means $\pm$ SD, n = 5-6. Equal loading of Western blots was ensured by β -actin. Means without a common letter differ ($P < 0.05$).

Figure 7. Schematic overview showing the analysed targets involved in the ECG-induced cell death in SW480 cells. Sharp arrows indicate positive inputs, whereas a line shows negative inputs.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A **B**

Figure 6

Figure 7