Research Article

Pharmacology

Pharmacology
DOI: 10.1159/000502076

Received: April 12, 2019
Accepted: July 12, 2019
Published online: August 21, 2019

Synergetic Effect of EP_1 Receptor Antagonist and (-)-Epigallocatechin-3-gallate in Hepatocellular Carcinoma

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Keywords
Epigallocatechin-3-gallate · Hepatocellular carcinoma · Selective EP_1 receptor antagonist

Abstract
Epigallocatechin-3-gallate (EGCG), the principal catechin of green tea, modulates different molecular mechanisms underlying hepatocellular carcinoma (HCC). Accumulating studies showed that the activation of prostaglandin (PG) receptor EP_1 promotes cell migration and invasion in different cancers, which could be inverted by blocking the EP_1 receptor. This study investigated the synergetic effects of EP_1-selective antagonist ONO-8711 and EGCG treatment on HCC to better understand the potential strategy to treat HCC. We found that EGCG significantly inhibited PGE_2 and EP_1-selective agonist induced migration of HCC cells and increased the ratio of Bax/Bcl-2 even in the presence of ONO-DI-004 or PGE_2. ONO-8711 significantly inhibited PGE_2-induced HCC proliferation while increased the inhibitory effect of EGCG on HCC cell viability and migration ability compared with EGCG alone. These findings suggest that a combination of ONO-8711 and EGCG is a potential treatment for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer. Although chemotherapy and radiotherapy are considered the principal approaches for HCC treatment, many patients receive a variety of treatments, given variability in outcomes. Recently, accumulating evidence has shown that Epigallocatechin-3-gallate (EGCG), an abundant bioactive ingredient in green tea, has cancer-chemopreventive effects [1, 2]. Particularly, EGCG, an antioxidant compound, induces apoptosis and inhibits tumor progression through its antioxidant and anti-inflammatory properties in several types of cancer [3, 4], including HCC [5]. Also, EGCG inhibits tumor growth directly through cell-cycle arrest and inducing apoptosis, and various proteins involved such as nuclear factor-kappa B [2, 6]. It is crucial to note that the suppression of nuclear factor-kappa B activation reduces the expression of cyclooxygenase-2 (COX-2) and prostaglandin (PG)E_2 production [7].

PGE_2 is catalyzed by COX-2, and it plays a key role in the pathogenesis of cancer, which leads by chronic inflammation [8, 9]. As a critical lipid mediator, PGE_2 binds to 4 identified EP receptors (EP_1, EP_2, EP_3, and EP_4) to

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exert its biological functions. PGE_2/EP_1 signaling pathway enhances HCC adhesion and migration through increasing the expression of survivin by activating EGFR and phosphorylation of focal adhesion kinase [10, 11]. EGFR phosphorylation and cell invasiveness induced by PGE_2 were blocked after EP_1 receptor knockdown by siRNA, or by the EP_1 receptor antagonist ONO-8711 [12]. Pharmacological and genetic studies show the selective EP_1 inhibitor significantly reduced colon cancer development, indicating this receptor plays a role in colon carcinogenesis [13]. A research showed that long-term administration of ONO-8711 in rat diet reduced azoxymethane-induced colon cancer incidence, multiplicity and volume of colon carcinomas [14]. One study showed that treatment with ONO-8713 significantly decreased PGE_2 levels and acute UVB-induced inflammation in skin [15]. Thus, these findings suggest that EP_1-selective antagonists seem to be promising candidates as chemotherapy alternatives, or supplements.

The anticancer mechanisms of EGCG in HCC are still not very clear. In our previous study, we found that both selective EP_1 agonist and PGE_2 significantly increased HCC cell growth and migration. In addition, EGCG suppressed EP_1 and PGE_2 expression, proliferation and migration that were induced by PGE_2 or EP_1 agonist [16]. Therefore, we hypothesize that a selective EP_1 receptor antagonist may increase the inhibitory effect of EGCG on HCC cell growth and migration. In the present study, we investigated the synergetic effects of ONO-8711 and EGCG on HCC cell migration and apoptosis to clarify a novel approach to HCC therapy.

Materials and Methods

Drugs

EGCG was purchased from Sigma (St Louis, MO, USA). EP1-receptor-antibody was obtained from Cayman Chemical, Ann Arbor, MI, USA. ONO-8711 and ONO-DI-004 were obtained from ONO Pharmaceutical Co, Osaka, Japan.

Cell Lines

HepG2 was purchased from Shanghai cell bank, Chinese Academy of Sciences. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO2/95% air.

Cell Proliferation Measurement

Proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (EZ4U; Biomedica, Vienna, Austria). HepG2 cells in exponential growth phase were seeded in a 96-well plate at a density of 8,000 cells per well and incubated overnight in DMEM medium supplemented with 10% FBS. Twenty-four hours of starvation in serum-free DMEM before cells were treated with PGE2 (4×10^{-6} M), ONO-8711 (210 nmol/L), ONO-DI-004 (400 nmol/L) or EGCG (12.5, 25, 50, 100 μg/mL). After treatment, 20 μL MTT solution (5.0 mg/mL in PBS) was added to each well, and the plates were placed in 37 °C for another 4 h. The formazan were dissolved in Dimethyl Sulfoxide (150.0 μL per well) and the plates were placed on a shaker at a low speed for 10 min at room temperature (RT). After that, the plates were read using a microplate reader (American Bio-Tek) at 490 nm. The inhibition of cell proliferation was calculated using the following formula: inhibition of cell proliferation (%) = (OD of the experimental samples – OD of blank control group)/(OD of the control – OD of blank control group) × 100% ($n = 6$, mean ± SD).

Transwell Filter Cell Migration Assay

The bottom of Boyden chambers is covered with polycarbonate filters with 8 μmol/L pore size (Costar, Bodenheim, Germany) were used as described previously [29]. The upper compartment was filled with serum-free DMEM cell suspension with a density of 5×10^5 cells/mL. DMEM medium containing 10% FBS was added to the lower compartment. After incubation for 12 h at 37 °C, the film was collected and it was found that cells adhered to the lower surface were fixed, stained and counted. The experiment was repeated 3 times with consistent results.

Wound Healing Assay

Cells were cultured in a 6-well plate and indicated at 37 °C until the cells were confluent. The yellow tip was used to scratch across the confluent cell layer to make a linear wound, and the cells were washed with PBS 3 times to remove dead cells. After that the cells were incubated with different compounds at 37 °C for 24 h and the pictures of the defined wound spot were taken with a microscope at 0 and 24 h. The area of the wound in the microscopic images was measured using Image J software (National Institutes of Health, MD). The relative distance of wound closure (%) = (distance at 0 h-distance at 24 h)/relative distance of control × 100%.

Immunofluorescence Microscopy

The coverslips were coated with collagen (25 μg/mL) and placed on the bottom of dishes. HepG2 cells climbed to coverslips and then were treated with different drugs. After that cells on coverslips were fixed with 4% paraformaldehyde in PBS for 30 min and blocked with 5% goat serum (Invitrogen, USA) in PBST (PBS supply with 0.1% Tween 20) for 30 min at RT and indicated with primary EP1-receptor antibody 2 h. Cells were washed with PBST 4 times, 5 min each time, and followed by a secondary antibody indication for 1 h. And the cells were washed 4 times again and mounted in Fluoromount G (Southern Biotechnology, USA). The cells were observed and pictured by Fluorescence Inversion Microscope System (Olympus Corporation, Japan), and measured by fluorescence spectrophotometers.

Western-Blot Analysis

Cells collection after treating with PGE2 or ONO-DI-004 for 2 h with or without an hour pretreatment with EGCG (100 μg/mL), and extracted protein with RIPA lysis buffer (Beyotime, China) contain 1% PMSF and 1% protease inhibitor (Bestbio, China). Concentration of protein was determined by Lowry Protein Assay. Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) was added to lysates at a ratio of 1:2, and then heated at 95 °C for 10 min. The proteins were separated by SDS-PAGE, and transferred to PVDF

membranes (Millipore, Bedford, MA, USA), followed by indicating with blocking buffer (5% nonfat dry milk in Tris-buffered saline contain 0.1% Tween 20) for 1 h. After that membranes were indicated with primary antibodies overnight at 4 °C and then indicated with appropriate HRP-conjugated secondary antibody at RT for 1 h. Immune complexes were performed with Enhanced chemiluminescence system (Pierce, Rockford, IL, USA) using hydrogen peroxide and luminol as substrate.

Cell Cycle and Cell Apoptosis Assay

Apoptotic cell death was analyzed by DNA fragmentation and total DNA. Cells were treated with different compounds for 24 h. Cells (1×10^6) were collected and fixed with 70% ethanol at –20 °C for 24 h and then centrifuged to remove supernatant; after that they were resuspended in 1 mL PBS containing RNase A (10 μg/mL) and incubated at 37 °C for 30 min. It was followed by staining with Propidium Iodide (PI, 40 μg/mL) for 30 min on ice, and then detected by Flow Cytometer with WinMDI software. Cells with subdiploid content were considered apoptotic.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5. All experiments were repeated at least 3 times. The results were presented as mean ± SE. Differences among different groups were analyzed using the non-parametric Kruskal-Wallis test. $p < 0.05$ was statistically significant.

Results

EGCG Inhibited PGE_2 or ONO-DI-004 Induced Proliferation and Migration in HCC Cells

The anti-migratory effect of EGCG on PGE_2 or ONO-DI-004-induced migration in HepG2 cell was measured by Transwell assay. Results showed that EGCG inhibited HepG2 migration in the presence of PGE_2 in a dose-dependent manner. Moreover, decreased ONO-DI-004 induced migration was observed after EGCG treatment (Fig. 1a–d). Besides, to observe the antiproliferative effect of EGCG in HepG2, cells were treated with a variety of EGCG concentrations analyzed by MTT assay. We found that EGCG significantly reduced cell viability in HepG2 cells (Fig. 1e; $p < 0.01$).

EGCG Inhibited PGE_2 or ONO-DI-004 Induced Anti-Apoptosis Effect in HepG2 Cells

EGCG treatment inhibited cell contact and enhanced the presence of early apoptotic morphology such as rounding off and chromatin condensation (Fig. 2a). Furthermore, apoptosis was measured after treating with EGCG, PGE_2 or ONO-DI-004 in HepG2 cells via Flow Cytometer. As a result, PGE_2 or ONO-DI-004 significantly inhibited HepG2 cell apoptosis, which could be inverted by EGCG (Fig. 2b).

EGCG Increased the Ratio of Bax/Bcl-2 Inhibited by PGE_2 or ONO-DI-004 in HepG2 Cells

Next, we investigated the effects of EGCG on Bcl-2, Bax and caspase-3 expression to more clearly understand that how EGCG affects apoptosis via PGE_2/EP_1 signaling. We found that Bcl-2 translation was significantly stimulated by PGE_2 or ONO-DI-004 in HepG2 cells (Fig. 3a). To the opposite, the ratio of Bax/Bcl-2 was significantly increased after EGCG treatment (Fig. 3b). In addition, caspase-3 expression was significantly lower after EGCG treatment compared with that of PGE_2 or ONO-DI-004 alone (Fig. 3c).

EGCG Induced Cell Cycle Arrest in the Presence of PGE_2 and ONO-DI-004 in HepG2 Cells

The effect of EGCG, PGE_2 and ONO-DI-004 on cell cycle progression in HepG2 cells was detected by flow cytometry. Results showed that normal cell cycle progression was disrupted after PGE_2 and ONO-DI-004 treatment with more cells in S phase compared with control group. Nevertheless, the percentage of G2/M phase cells increased from 13.7 to 23.7% with EGCG treatment compared with control group. Besides, EGCG induced cell cycle arrest in the G2/M phase in the presence of either PGE_2 or ONO-DI-004 (Table 1, $p < 0.01$).

The Combination of EGCG and ONO-8711 Inhibited Proliferation and Migration in HepG2 Cell

Here, we determined whether the EP_1 receptor plays a role in PGE_2-induced HCC carcinogenesis by measuring the effects of EP_1 antagonist ONO-8711 on HepG2 cells in the presence of PGE_2 by MTT assay. Results showed that ONO-8711 inhibited PGE_2-induced proliferation of HepG2 cells (Fig. 4a). Furthermore, we designed to test effects of combination of EGCG and ONO-8711 on cell proliferation. Treatment with the combination of EGCG and ONO-8711 resulted in significantly additional inhibitory effects on HepG2 cells proliferation (Fig. 4b; $p < 0.01$). In addition, we investigated whether combination treatment had an additive effect on HCC cell migration measured by a wound healing assay. Results demonstrated that combination treatment had an additional effect on HepG2 cell migration compared with either treatment alone (Fig. 4c, d).

The Combination of EGCG and ONO-8711 Reduced EP_1 Receptor Expression

We determined whether the expression of EP_1 receptor was altered after treating with EGCG and ONO-8711

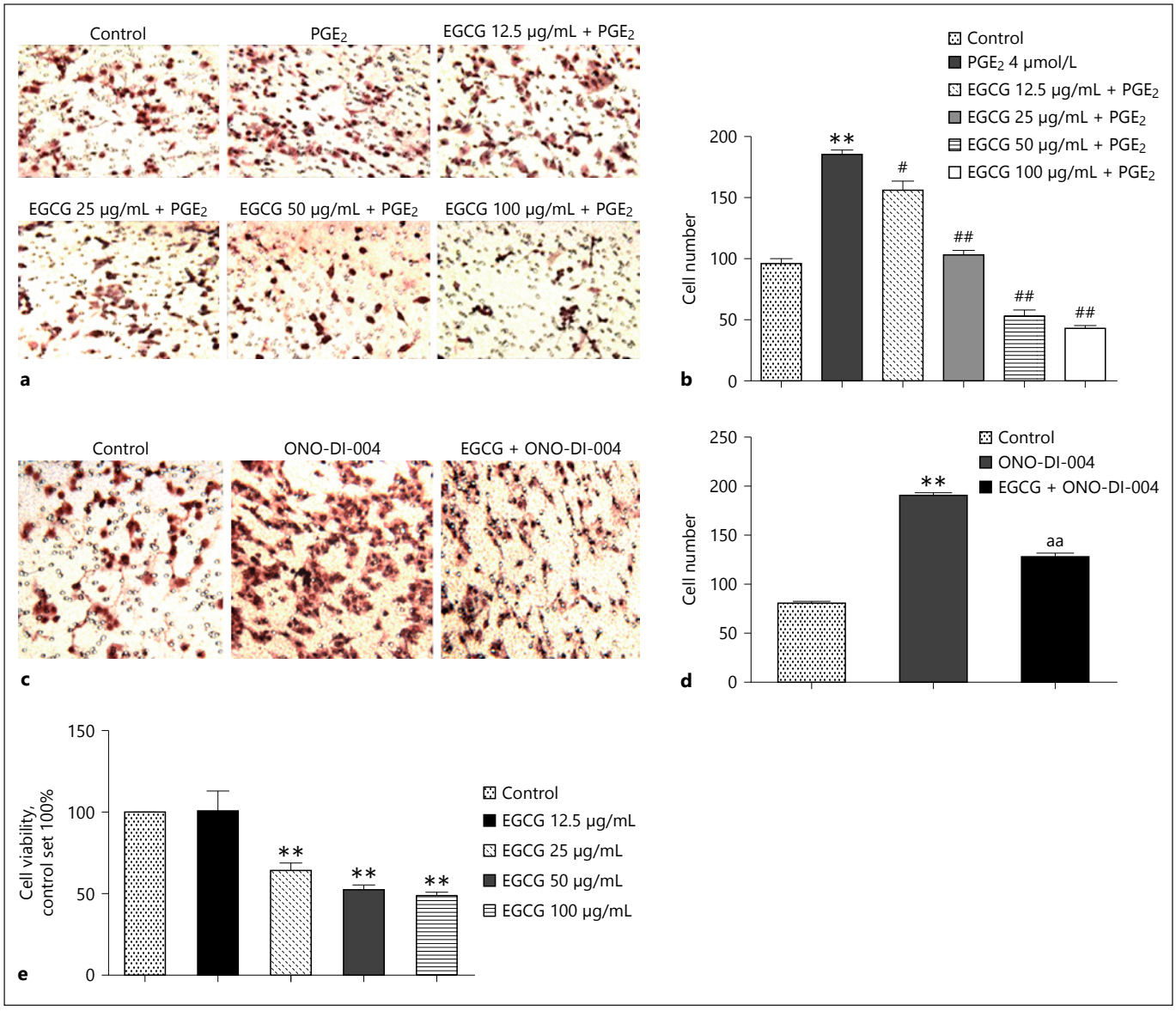

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Fig. 1. EGCG inhibited PGE_2 or ONO-DI-004 induced proliferation and migration in HepG2 cells. **a**, **c** Representative photographs of invading cells by Transwell filter assay are shown. **b** The effects of EGCG on PGE_2-induced migration of HepG2 cells. ** $p < 0.01$ compared with control group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with PGE_2 (4 μmol/L) group. **d** EGCG treatment in ONO-DI-004-induced HepG2 cells. ** $p < 0.01$ compared with control group; ^{aa} $p < 0.01$ compared with ONO-DI-004 treatment group. **e** The effect of EGCG on HepG2 cells viability. Cells were treated with differing concentrations of EGCG (0, 12.5, 25, 50, 100 μg/mL) in serum-free medium for 24 h and analyzed by MTT assay. ** $p < 0.01$ compared with control group. Data shown are expressed as the mean ± SEM of 3 separate experiments, each of which was performed in duplicate. PGE_2, prostaglandin E_2; EGCG, epigallocatechin-3-gallate.

detected by Immunofluorescence microscopy. First, we investigated the action of ONO-DI-004 on EP_1 expression at different time points. We found that the expression of EP_1 protein was remarkably increased at 2 h treated with ONO-DI-004 (Fig. 5a). Therefore, protein expression was detected at 2 h after treatment in the further test. Indeed, results showed EP_1 protein expression was significantly enhanced after treatment with PGE_2 or ONO-DI-004, but decreased in combination treatment of EGCG and ONO-8711 (Fig. 5b, $p < 0.01$).

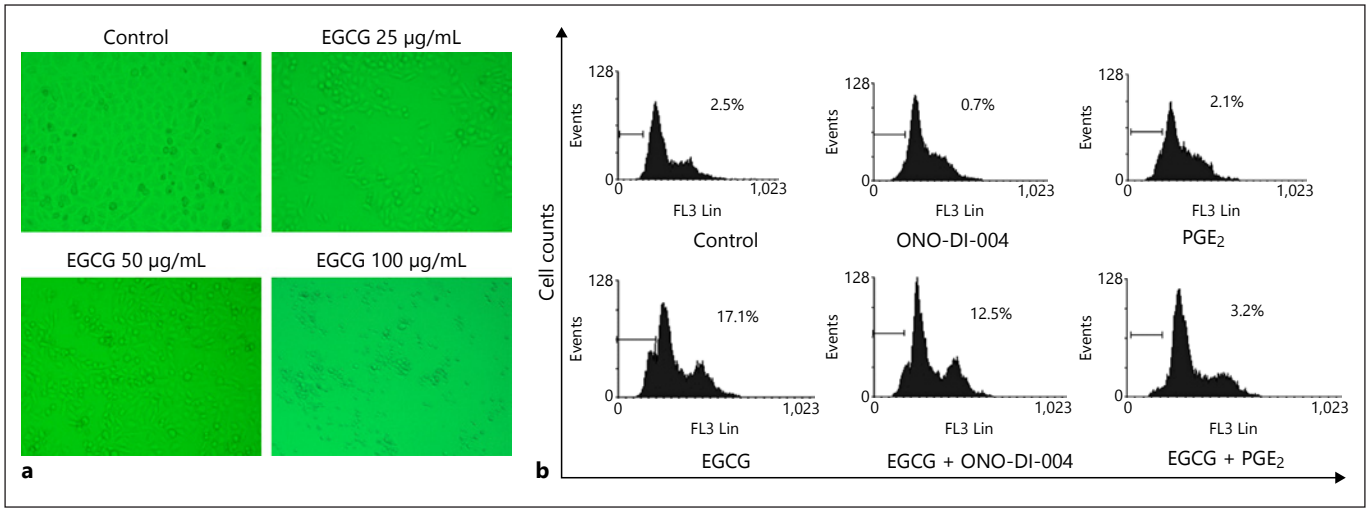

Fig. 2. EGCG inhibited PGE_2 or ONO-DI-004 induced anti-apoptosis effect in HepG2 cells. **a** Apoptosis was evaluated by microscopic analysis. Compared with the control group, EGCG treatment 100 μg/mL inhibited cell contact and development of early apoptotic morphology and chromatin condensation. **b** Apoptosis was evaluated by PI staining. The percentage of cells undergoing apoptosis was defined as the ratio of apoptotic cells to whole cells. EGCG, epigallocatechin-3-gallate; PGE_2, prostaglandin E_2.

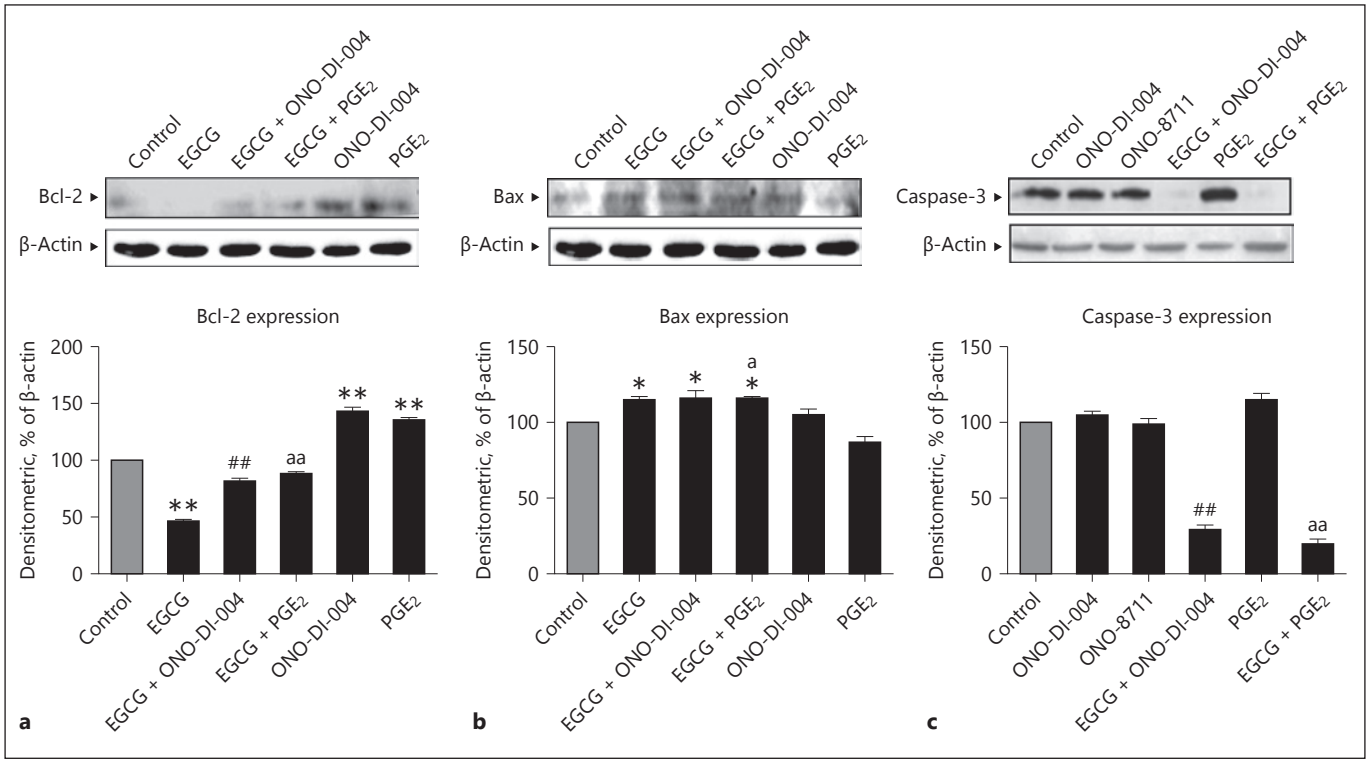

Fig. 3. EGCG increased the ratio of Bax/Bcl-2 inhibited by PGE_2 or ONO-DI-004 in HepG2 cells. Whole cell lysates were analyzed by Western blotting. **a** EGCG-mediated bcl-2 expression in HepG2 cells. **b** EGCG-mediated Bax expression in HepG2 cells. **c** EGCG-mediated caspase-3 activation in HepG2 cells. * $p < 0.05$, ** $p < 0.01$ compared with the control group; ## $p < 0.01$ compared with ONO-DI-004 group; a $p < 0.05$, aa $p < 0.01$ compared with the PGE_2 group. Data shown are expressed as the mean ± SEM of 3 separate experiments, each of which was performed in duplicate. EGCG, epigallocatechin-3-gallate; PGE_2, prostaglandin E_2.

Table 1. The effects of EGCG on cell cycle arrest in HepG2 cells

Group	Cell cycle phrase, %		
	G0/G1, mean ± SD	S, mean ± SD	G2/M, mean ± SD
Control	67.5±1.96	14.7±3.43	13.7±2.14
ONO-DI-004	61.74±4.28	21.7±2.93**	15.27±1.48
PGE_2	63.77±1.07	22.6±1.97**	10.58±4.28
EGCG	50.59±3.25**	9.43±1.94**	23.7±3.19**
EGCG + ONO-DI-004	51.36±1.53##	10.44±2.92##	25.67±2.16##
EGCG + PGE_2	67.48±2.61	8.98±1.64$$	20.3±1.73$$

Cells were cultured with PGE_2, ONO-DI-004, EGCG, EGCG + ONO-DI-004, or EGCG + PGE_2. Data are presented as percentage of cells in given cell cycle phase.

** $p < 0.01$ compared with the control group.

$p < 0.01$ compared with the ONO-DI-004 group.

$$ $p < 0.01$ compared with the PGE_2 group.

EGCG, epigallocatechin-3-gallate; PGE_2, prostaglandin E_2.

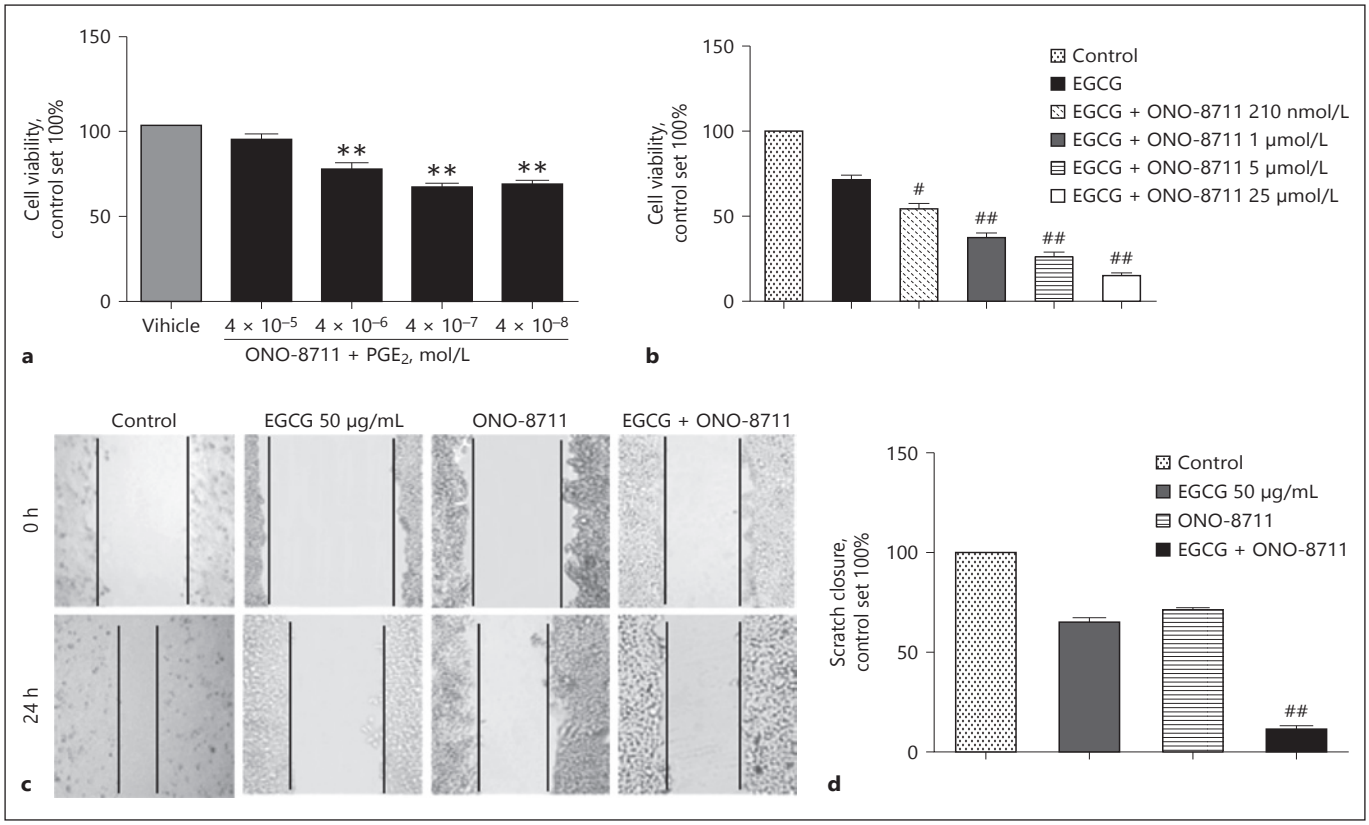

Fig. 4. The combination of EGCG and ONO-8711 inhibited proliferation and migration in HepG2 cell. **a** ONO-8711 inhibited PGE_2-induced HepG2 cell proliferation. HepG2 cells were treated with differing concentrations of PGE_2 (4×10^{-5}, 4×10^{-6}, 4×10^{-7}, 4×10^{-8} M) in serum-free medium with ONO-8711 (210 nmol/L) for 24 h analyzed by MTT assay. **b** HepG2 cells were treated with various concentrations of ONO-8711 (0, 210 nmol/L, 1, 5, 10 μmol/L) with or without EGCG (50 μg/mL) in serum-free medium for 24 h then analyzed by MTT assay. **c** Representative photographs (×100) of invading cells by wound-healing assay. **d** Effect of EGCG (50 μg/mL) and ONO 8711 (210 nmol/L) on HepG2 cell scratch closure. Data shown are expressed as the mean ± SEM of 3 separate experiments, each of which was performed in duplicate. ** $p < 0.01$ compared with untreated group. # $p < 0.05$ and ## $p < 0.01$ compared with EGCG (50 μg/mL). PGE_2, prostaglandin E_2; EGCG, epigallocatechin-3-gallate.

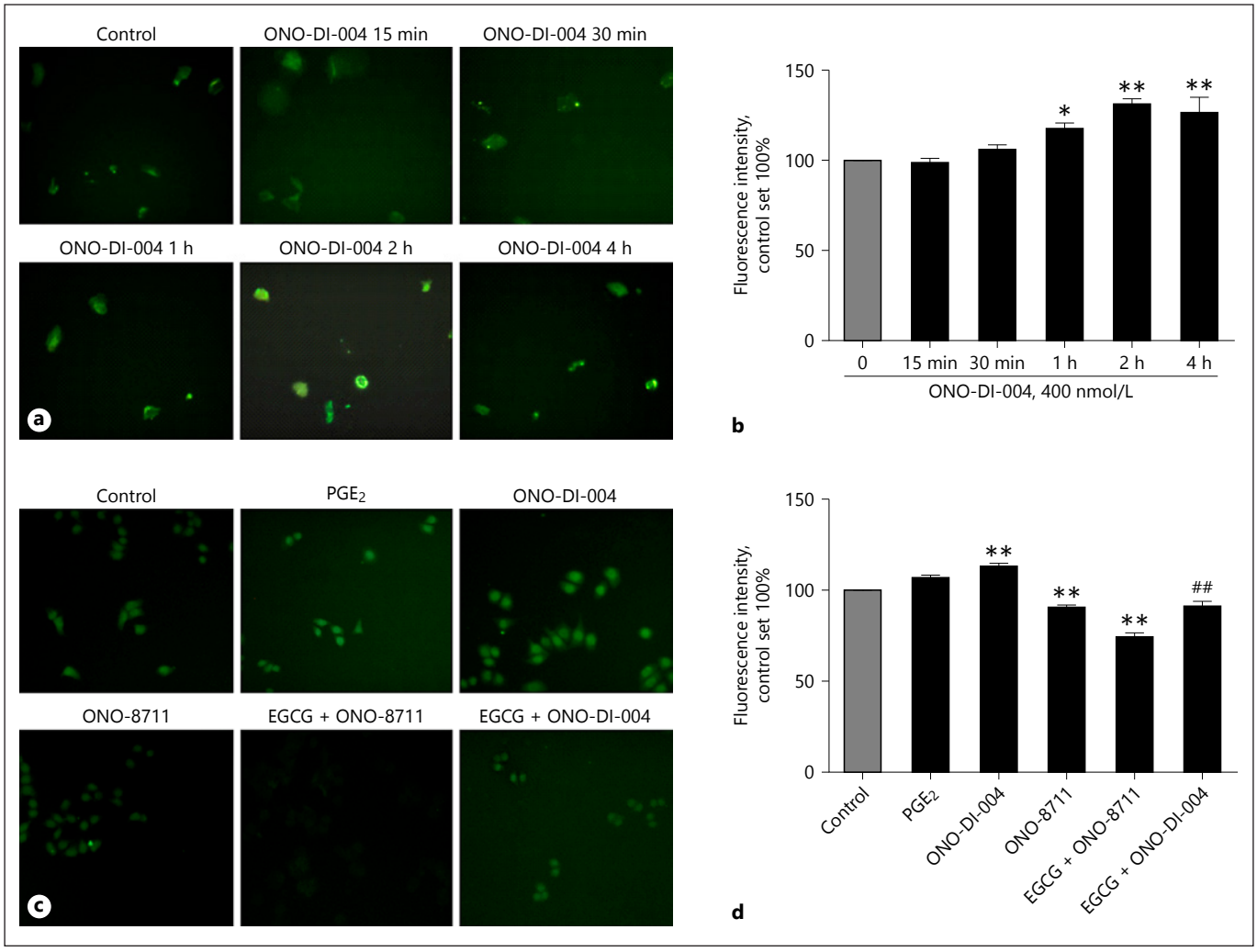

Fig. 5. The combination of EGCG and ONO-8711 inhibited EP_1 receptor expression in HepG2 cells. **a**, **b** The effect of ONO-DI-004 on EP_1 expression at different time. * $p < 0.05$, ** $p < 0.01$ compared with control groups. **c**, **d** EP_1 receptor expression was measured after 2 h of incubation by immunofluorescence microscopy. The data represent 3 independent experiments. Data shown are expressed as the mean ± SEM of 3 separate experiments, each of which was performed in duplicate. ** $p < 0.01$ compared with control groups. ## $p < 0.01$ compared with ONO-DI-004 group. PGE_2, prostaglandin E_2; EGCG, epigallocatechin-3-gallate.

Discussion

The compounds in green tea mainly consist of epigallocatechin gallate (EGCG), caffeic acid, gallic acid, catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin, C gallate, EC gallate, and GC gallate. EGCG is the major C in tea. One previous study demonstrated that the antiproliferative effects of GC, epigallocatechin, GC gallate, and EGCG were stronger than their corresponding compounds C, EC, C gallate, and EC gallate respectively [17]. Besides, EGCG is the most effective cancer chemopreventive polyphenol in green tea [17].

The present study demonstrated that EGCG inhibited PGE_2 and ONO-DI-004 induced HCC migration and proliferation, as well as promoted HCC apoptosis. In addition, increased ratio of Bax/Bcl2 but decreased EP_1 protein expression was observed after EGCG treatment. Furthermore, the synergistic effect of EGCG and ONO-8711 showed in suppressing proliferation and migration of HCC cells.

Apoptosis, a form of programmed cell death, plays a crucial role in the maintenance of cellular and tissue homeostasis [18]. The proteins of the Bcl-2 family are essential regulators of apoptosis, such as Bcl-2 and Bax [19]. A previous study observed that EGCG induced

apoptosis by downregulated Bcl-2 and upregulated miR-16 in HLE cells [20]. In the current study, we found that EGCG induced apoptosis and down-regulated Bcl-2 and caspase-3 even in the presence of PGE_2 or EP_1 agonist in HepG2 cells. In addition, Bax protein was upregulated. These findings suggest that EGCG induces apoptosis by increasing the ratio of Bax/Bcl-2. The results are consistent with a previous research that showed caspase-3/7 activity decreased after EGCG treatment [21]. However, some data show caspase-3 activation after EGCG treatment [22]. The conflicting results may be explained by differing cell phase timing and cell lines. Further studies are needed to clarify these findings. Together, these results indicate that EGCG induce apoptosis in a variety of ways in HCC cells, but apoptosis of HepG2 cells was induced mainly by targeting Bcl-2 and upregulating Bax.

The cell cycle is regulated precisely to avoid proliferation under adverse conditions. Cells are arrested in G1, S and G2/M phases to prevent replication of damaged DNA or aberrant mitosis. One recent study demonstrated that EGCG functionalized chitin derivative inhibited more HepG2 cells in the G2/M phase [23]. Consistently, in the current study, the result showed that cells were arrested in the G2/M phase by treating with EGCG in the presence of PGE_2 and ONO-DI-004 or not. However, another study observed that EGCG arrested SMMC7721 cells at S phase [24]. The conflict result might be attributed to the unique features of HCC cell lines.

Accumulating studies have showed that EP receptors are important in the development of other types of cancers, including colon and breast cancer, and they highlight that the specific EP receptor antagonists were effective when they are used as chemopreventive agents [25, 26]. EGCG modulates a variety of molecular pathways in HCC, including inhibiting expression of COX-2 and mPGES [27, 28]. Our previous study showed that EGCG downregulated PGE_2 in HCC cells. Moreover, we found that the growth and migration of HCC cells in vitro were decreased after treating with ONO-8711 by blocking the EP_1 receptor [16]. In the current study, EGCG could suppress PGE_2 or ONO-DI-004 induced migration and anti-apotosis. Moreover, synergetic anti-proliferative and anti-migratory effects of ONO-8711 and EGCG were observed. Those findings suggested that the regulation of PGE_2-EP_1 signaling plays an important role in HCC. Clearly, the detailed mechanism is not understood, and more work is needed.

In conclusion, our study provides evidence that the combination of EGCG and EP_1 receptor antagonist inhibited HCC cell growth by affecting cell growth, migration, and cell cycle as well as inducing apoptosis via downregulation of PGE_2/EP_1 activity. The results suggested that the combination of EGCG and EP_1 receptor antagonist might be a potential anticancer strategy in HCC therapy.

Disclosure Statement

The authors have no conflicts of interest to disclose.

Funding Sources

This work is supported by Natural Science Foundation of Colleges and Universities in Anhui Province (KJ2017A179).

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