A combination of eicosapentaenoic acid-free fatty acid, epigallocatechin-3-gallate and proanthocyanidins has a strong effect on mTOR signaling in colorectal cancer cells

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Colorectal cancer (CRC) is one of the major causes of cancer death worldwide. The development of novel anti-CRC agents able to overcome drug resistance and/or off-target toxicity is of pivotal importance. The mammalian target of rapamycin (mTOR) plays a critical role in CRC, regulating protein translation and controlling cell growth, proliferation, metabolism and survival. The aim of this study was to explore the effect of a combination of three natural compounds, eicosapentaenoic acid-free fatty acid (EPA-FFA), epigallocatechin-3-gallate (EGCG) and proanthocyanidins (grape seed [GS] extract) at low cytotoxic concentrations on CRC cells and test their activity on mTOR and translational regulation. The CRC cell lines HCT116 and SW480 were treated for 24h with combinations of EPA-FFA (0–150 μM), EGCG (0–175 μM) and GS extract (0–15 μM) to evaluate the effect on cell viability. The low cytotoxic combination of EPA-FFA 150 μM, EGCG 175 μM and GS extract 15 μM completely inhibited the mTOR signaling in HCT116 and SW480 cells, reaching an effect stronger than or comparable to that of the mTOR inhibitor Rapamycin in HCT116 or SW480 cells, respectively. Moreover, the treatment led to changes of protein translation of ribosomal proteins, e-Myc and cyclin D1. In addition, we found a reduction of clonal capability in both cell lines, with block of cell cycle in G1/G0 and induction of apoptosis. Our data suggest that the low cytotoxic combination of EPA-FFA, EGCG and GS extract, tested for the first time here, inhibits mTOR signaling and thus could be considered for CRC treatment.

Introduction

Colorectal cancer (CRC) is one of the leading causes of death worldwide in both men and women (1). Antineoplastic drugs are widely used for CRC treatment but drug resistance and/or off-target toxicity limit their efficiency (2,3). Therefore, the development of novel anti-CRC agents is strikingly important.

Abbreviations: CRC, colorectal cancer; EPA-FFA, eicosapentaenoic acid-free fatty acid; EGCG, epigallocatechin-3-gallate; GS, grape seed; mRNA, messenger RNA; mTOR, mammalian target of rapamycin.

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The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that regulates protein translation and controls key cellular processes such as cell metabolism, growth, proliferation, angiogenesis and survival, by integrating signals from growth factors, nutrients and energy status (4,5). It is known that mTOR, acting downstream of the phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/Akt signaling, forms two multiprotein complexes: mTORC1, sensitive to Rapamycin, and mTORC2, resistant to Rapamycin, which have distinct physiological functions. In particular, mTORC1 regulates messenger RNA (mRNA) expression, ribosome biogenesis, cellular growth, proliferation and survival (4,6). In response to mitogenic stimuli or nutrient availability, mTORC1 is activated, leading to phosphorylation of two well-characterized downstream effectors: the 70kDa ribosomal protein S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1), which together control protein synthesis (7).

mTOR is an oncogene that plays a pivotal role in several human cancers (8–10). Emerging data have shown that components of the mTOR signaling pathway are frequently activated or overexpressed in CRC, representing a promising therapeutic target (5,11,12).

Rapamycin and its analogs (rapalogs) like RAD001 (everolimus Novartis), AP23573 (Ariad Pharmaceuticals) and CCI779 (temsirolimus) are the most well-studied mTORC1 inhibitors. One problem regarding the use of this class of drugs for cancer treatment is related to a distinct spectrum of dose-limiting toxicities (13) such as skin reactions, mucusitis and myelosuppression (11,14).

In nature, fruits and vegetables are an important source of phytochemicals which have chemopreventive activities; however, they can also be suitable for cancer treatment (15). In particular, epigallocatechin-3-gallate (EGCG), the most important polyphenol from green tea, inhibits cell growth and proliferation in vitro, inducing apoptosis on CRC cells (16,17).

Another important source of phytochemicals, in particular proanthocyanidins, is grape seed (GS) extract. Data indicate that GS extract has strong growth inhibitory and apoptosis-inducing effects in CRC cell lines (18,19) and xenografts (20). Fish oil is a source of omega-3 (ω-3) polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA). EPA inhibits cell proliferation and tumor growth in murine and rodent models (21,22). Moreover, EPA has an effect in reducing the size of liver metastases in animal models (23–25). Overall, literature data show that each of the three compounds mentioned above, tested as single molecules, have strong effects on CRC cells (26–28), and EGCG and EPA have been found to act on the PI3K/Akt/mTOR pathway (29,30). However, to achieve anticancer effects, dosages are usually high. This would make natural compounds toxic rather than beneficial.

To minimize the toxic effects produced by the use of single agents at high concentrations and to develop a strategy more suitable in humans, in this study, we tested for the first time a combination of EPA as free fatty acid (FFA), EGCG and GS extract at low toxicity dosage, on mTOR signaling and on translational regulation in CRC cell lines. We found that this combination completely inhibited the mTOR signaling, led to changes of protein translation, reduced cell proliferation and induced apoptosis in CRC cells.

Materials and methods

Cell lines and treatments

The human CRC cell lines HCT116 (PIK3CA mutant, Rapamycin sensitive, p53 wild-type) and SW480 (PIK3CA wild-type, Rapamycin resistant, p53 mutant) were obtained from ATCC (Manassas, VA) and cultured in IMDM

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supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM glutamine (Euroclone, Milan, Italy). Cells were maintained at 37°C and 5% CO₂. In our laboratory, all cells are tested and authenticated every year using known genetic and epigenetic markers.

Eicosapentaenoic acid-free fatty acid (EPA-FFA, ALFA, SLA Pharma AG, Switzerland), EGCG (purity ≥95%, Sigma-Aldrich, Milan, Italy), GS (purified at a local market) extract and Rapamycin (Cell Signaling Technology, Milan, Italy) were used for cell culture treatments.

Preparation of GS extracts

One hundred grams of Aglianico red GSs were freeze dried and finely ground in a blender. They were then extracted three times with 200mL of methanol obtaining the crude extract that was analyzed by liquid chromatography coupled with tandem mass spectrometry, as described previously (31). The main compounds identified were catechins, dimers and trimers, although significant amounts of polymers, up to 8 units, were also detected. To obtain the molar concentration of GS extract, the sum of all peaks present in the chromatogram were considered and quantified using the calibration curve of procyanidin B2. Therefore, the GS amount was expressed in procyanidin B2 equivalents. In particular, GS extract 10 mmol/l procyanidin B2 equivalents were prepared and opportunistically diluted for cell treatments.

Cell viability assay

Cells were seeded into 96-well plates (3 × 10³ cells/well) and treated with different concentrations of EPA-FFA (0–300 µM), EGCG (0–400 µM) and GS (0–200 µM) in combination. After 24–96 h treatment, the number of viable cells compared with the control was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT, Sigma-Aldrich) following the manufacturer’s instructions. Each experiment was performed in quintuplicate and repeated independently twice.

Colony-forming assay

Cells were seeded in six-well plates (10⁴ cells/well) and treated with a combination of EPA-FFA 150 µM, EGCG 175 µM and GS 15 µM or Rapamycin 20nM for 24h. After colony formation, the plates were washed with phosphate-buffered saline and fixed with 0.5% crystal violet in paraformaldehyde. The total number of visible colonies was counted using ImageJ software (rsweb.nih.gov/ij). Each experiment was performed in triplicate and repeated independently three times.

Cell cycle analysis

Upon treatment with EPA-FFA 150 µM, EGCG 175 µM and GS 15 µM or Rapamycin 20nM for 24h, cells were trypsinized, pelleted and resuspended in the Nusse 1 solution (NaCl 584 mg/L, trisodium citrate 1139 mg/L, ribonuclease 10 mg/L and Nonidet P40 300 µg/ml) to obtain a final concentration of 2 × 10⁶ cells/ml and incubated for 45 min on ice. An equal amount of Nusse 2 solution (sucrose 15.5 g/L and citric acid 15 g/L) was then added to the mixture to obtain a final concentration of 10⁶ cells/ml. Samples were stained with propidium iodide (50 µg/ml) for 15 min and analyzed by flow cytometry (FACSaria, BD Biosciences, Milan, Italy).

Apoptosis analysis

Cells were treated for 24h with the combination of EPA-FFA 150 µM, EGCG 175 µM and GS 15 µM or Rapamycin 20nM. Apoptosis was evaluated with the DeadEnd fluorometric TUNEL System (Promega, Milan, Italy), according to the manufacturer’s recommendations. The apoptotic index was expressed as the number of apoptotic cells/total cells.

Western blot analysis

Cells were treated with EPA-FFA, EGCG and GS as single compounds or in combination or with Rapamycin 20nM for 4–8h. These time points were chosen because in the literature the effects on mTOR targets are studied between 1 and 8 h of treatment (32). Fifty micrograms of proteins for each sample were analyzed on 12% sodium dodecyl sulfate–polyacrylamide gel (Life Technologies, Monza, Italy) and transferred onto nitrocellulose membrane. After blocking, membranes were incubated overnight at 4°C with primary antibodies: phosphorylated p70S6K (Thr389) (1:1000), total p70S6K (1:800) and 4EBP1 (1:600) (Cell Signaling Technology), phosphorylated 4E-BP1 (Ser65/Thr70) (1:1000), cyclin D1 (1:500) and c-Myc (1:500) (Santa Cruz antibodies: phosphorylated p70S6K (Thr389) (1:1000), total p70S6K (1:800) and 4EBP1 phosphorylation at 4 h. Importantly, the inhibitory effect on cell viability persisted for 24 h in the absence of the combination (Supplementary Figure 1, available at Carcinogenesis Online).

We then evaluated the phosphorylation status of two well-known mTOR downstream effectors, p70S6K and 4EBP1, in treated cells for 4h on HCT116 cells with EPA-FFA and EGCG used as single compounds decreased the phosphorylation of p70S6K, whereas GS increased or did not change the levels of this protein. However, the phosphorylation of 4EBP1 did not seem to be significantly affected when all three natural substances were used as single compounds. Importantly, the combination of EPA-FFA+EGCG+GS caused a decrease in the number of viable cells compared with the control in HCT116 (P < 0.01) and SW480 (P < 0.001) cells, respectively (Figure 1A and B left). In addition, we tested the effect of the combination of EPA-FFA+EGCG+GS on HCT116 and SW480 cells at 48, 72 and 96h, finding a significant decrease at all the time points tested compared with the control (P < 0.001 for both HCT116 and SW480), Importantly, the inhibitory effect on cell viability persisted for 24 h in the absence of the combination (Supplementary Figure 1, available at Carcinogenesis Online).

Results

Treatment with EPA-FFA, EGCG and GS decreases mTOR activity in CRC cells

To investigate the effect of EPA-FFA, EGCG and GS on cell viability, we treated HCT116 and SW480 cells with different concentrations of the compounds in combination for 24h. We found that the combination with the treatment of EPA-FFA 150 µM, EGCG 175 µM and GS 15 µM (hereafter named as EPA-FFA+EGCG+GS) caused a decrease in the number of viable cells compared with the control in HCT116 (P < 0.01) and SW480 (P < 0.001) cells, respectively (Figure 1A and B left). In addition, we tested the effect of the combination of EPA-FFA+EGCG+GS on HCT116 and SW480 cells at 48, 72 and 96h, finding a significant decrease at all the time points tested compared with the control (P < 0.001 for both HCT116 and SW480). Importantly, the inhibitory effect on cell viability persisted for 24 h in the absence of the combination (Supplementary Figure 1, available at Carcinogenesis Online).
versus combination) comparable to Rapamycin (P < 0.01 control versus Rapamycin) and a decrease of P-p70S6K compared with control, even though this latter did not reach statistical significance (Figure 1B right).

In addition, we tested whether the inhibitory effect of EPA-FFA+EGCG+GS for 4 h on mTOR targets persisted after the removal of the treatment. Both in HCT116 and SW480 cells, the modulation of p70S6K and 4EBP1 phosphorylation was lost 20 h after the removal of the combination (Supplementary Figure 3, available at Carcinogenesis Online). These results indicate that the combined treatment with EPA-FFA 150 µM, EGCG 175 µM and GS 15 µM at low toxicity concentrations for 4 h inhibits mTOR signaling in HCT116 and SW480 cell lines, as shown by inhibition of its downstream effectors.

**EPA-FFA, EGCG and GS treatment reduces translation of ribosomal proteins, c-Myc and cyclin D1 in CRC cells**

Because mTOR regulates the initiation of translation, we next examined the effects of EPA-FFA+EGCG+GS for 4 h on translational regulation through the analysis of polysome profiles in HCT116 (Figure 2A). The sum of absorbance at 260 nm of polysomal fractions was lower in the treated sample compared with the control, but this difference was not statistically significant. However, when we evaluated the levels of selected mRNAs known to be translationally regulated by mTOR, such as those encoding for ribosomal proteins (L5, L11, L13), c-Myc and cyclin D1, we found a significant decrease of L5 in total RNA (P = 0.03), L11 (P = 0.03) and cyclin D1 (P = 0.005) in RNA associated with polysomal fractions (Figure 2B) (33,34). In addition, the same analysis was performed in HCT116 cells treated with Rapamycin 20 nM for 4 h. No statistically significant difference was observed between the polysomal profiles of control and Rapamycin-treated cells (Supplementary Figure 4A, available at Carcinogenesis Online). We then evaluated total and polysomal levels of selected mRNAs encoding for ribosomal proteins in Rapamycin-treated cells finding a significant decrease only for L13 in total RNA (P = 0.03 control versus Rapamycin, Supplementary Figure 4B, available at Carcinogenesis Online). To identify the mRNAs that were subjected to transcriptional and/or translational regulation after the treatment with EPA-FFA+EGCG+GS, the relative translatability of mRNA was determined by normalizing the change in abundance in polysomal RNA to the change in abundance in total RNA for each mRNA (Poly/Total) (Table 1). As reported previously (35), mRNAs with a Poly/Total ratio of 0.65 or lower were considered as translationally decreased whereas those mRNAs with a Poly/Total ratio equal or higher to 1.5 were considered translationally increased. Our data suggest that the treatment with EPA-FFA+EGCG+GS significantly decreased the total mRNA abundance of L5 and reduced the translation of L11, L13, c-Myc and cyclin D1.

We then investigated whether the observed translational decrease of c-Myc and cyclin D1 mRNA might affect their protein levels. Compared with the control, in treated HCT116 cells, we found suppression of c-Myc (P < 0.05) and a decrease of cyclin D1 that, however, did not reach statistical significance. Notably, the treatment with Rapamycin did not affect the level of c-Myc and cyclin D1 compared with the control (P = n.s.), (Figure 3A). In addition, we assessed the effect of EPA-FFA+EGCG+GS or Rapamycin on c-Myc and cyclin D1 proteins in SW480 cells, without finding any difference from the control (P = n.s.) (Figure 3B).

**EPA-FFA, EGCG and GS treatment inhibits cell proliferation and increases apoptosis in CRC cells**

To understand whether the reduction of c-Myc and cyclin D1 could cause alterations in cell proliferation, we examined the effect of EPA-FFA+EGCG+GS treatment for 24 h on the clonogenic capability of HCT116 and SW480 cells (Figure 4A). We found a significant reduction of the number of colonies in HCT116 and SW480 cells treated with the combination (P < 0.001 and P < 0.01, respectively). No significant reduction in the number of colonies was obtained by treating cells with Rapamycin 20 nM for 24 h.
In addition, we performed a cell cycle analysis in both cell lines after treatment with the combined EPA-FFA+EGCG+GS mixture or with Rapamycin 20nM for 24h. As shown in Figure 4B, in HCT116, the combined mixture significantly increased cells in the G0G1 phase and reduced cells in the S and G2M phases (P < 0.01 control versus combination), whereas Rapamycin did not change the distribution of cells in the different cell cycle phases (P = n.s. control versus Rapamycin). Conversely, the treatment with either the combination or Rapamycin did not cause alteration of the cell cycle in SW480 cells (P = n.s.). We then evaluated whether treatment with EPA-FFA+EGCG+GS affects apoptosis, and we found a significant increase in the apoptotic index both in HCT116 (P < 0.05 control versus combination) (Figure 4C) and SW480 cells (P < 0.001 control versus combination). Furthermore, we assessed apoptosis on HCT116 and SW480 cells treated with Rapamycin, finding no differences compared with untreated control cells (P = n.s.).

Our data indicate that this low cytotoxic combination has antiproliferative and proapoptotic effects in CRC cell lines. In particular, EPA-FFA+EGCG+GS treatment was more effective than Rapamycin in the tested CRC cell lines and this is in line with the effects observed at the molecular levels described above.

**Discussion**

In this study, we have shown that the treatment with a combination of three natural compounds, EPA-FFA, EGCG and GS, at low cytotoxic concentrations, decreased mTOR activity in CRC cell lines leading to a reduced translation of the c-Myc and cyclin D1 cancer genes. These effects were stronger than those obtained with the selective mTOR inhibitor Rapamycin. In addition, the EPA-FFA, EGCG and GS treatment affected proliferation with a block of cells in G0G1 phase and increased apoptosis. The mTOR pathway is a central regulator of many cellular events, such as proliferation, growth, metabolism and survival and is frequently activated in CRC, due to a gain of function mutation of the PI3K catalytic subunit gene (36–38). With its biological role and its constitutive activation in CRC, mTOR has become a key target of cancer research and drug development. Several studies have shown that pharmacological inhibitors of mTOR reduce the growth of CRC cells *in vitro* and *in vivo*, but clinical studies highlight drawbacks related to toxicity that could lead to treatment failure (11,13,14). Moreover, mutations in the PI3K catalytic subunit that confer resistance to some inhibitors have been identified (39).
Fig. 3. Effect of EPA-FFA, EGCG and GS on c-Myc and cyclin D1. c-Myc and cyclin D1 proteins levels were assessed in cell extracts treated with EPA-FFA+EGCG+GS or Rapamycin from (A) HCT116 or (B) SW480 cells. Analyses were performed on logarithmic transformed data for HCT116. After the ANOVA global test ($P = 0.0190$, $n = 4$, HCT116; $P = \text{n.s.}$, $n = 4$), Tukey’s post hoc test was used for pairwise comparisons.

Fig. 4. Effect of EPA-FFA, EGCG and GS on cell proliferation and apoptosis in HCT116 and SW480 cells. (A) Clonogenic assay on HCT116 and SW480 cells treated with EPA-FFA+EGCG+GS or Rapamycin (ANOVA $P = 0.0012$ and $P = 0.0017$ for HCT116 and SW480, respectively; Tukey’s test was applied as post hoc test, $n = 3$). (B) Cell cycle analysis (ANOVA $P = 0.0059$ for HCT116 and $P = \text{n.s.}$ for SW480, $n = 3$, Dunnett’s test) (C) Tunnel assay: representative pictures and quantification. Statistical significance was tested using one-way ANOVA ($P = 0.0447$ for HCT116 and $P = 0.0011$ for SW480, followed by Dunnett’s test for comparison with the control cells, $n = 2$). Ctrl = control; Comb = Combination; Rapa = Rapamycin.
Effect of EPA-FFA, EGCG and GS on mTOR signaling in CRC

Supplementary material
Supplementary Figures 1–4 and Table I can be found at http://carcin.oxfordjournals.org/

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