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ORIGINAL ARTICLE

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Docosahexaenoic acid enhances the susceptibility of human colorectal cancer cells to 5-fluorouracil

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Abstract *Purpose*: Powerful growth-inhibitory action has been shown for n-3 polyunsaturated fatty acids against colon cancer cells. We have previously described their ability to inhibit proliferation of colon epithelial cells in patients at high risk of colon cancer. In the work reported here we investigated the ability of docosahexaenoic acid (DHA) to potentiate the antineoplastic activity of 5-fluorouracil (5-FU) in p53-wildtype (LS-174 and Colo 320) and p53-mutant (HT-29 and Colo 205) human colon cancer cells. *Methods*: When in combination with DHA, 5-FU was used at concentrations ranging from 0.1 to 1.0 μM, much lower than those currently found in plasma patients after infusion of this drug. Similarly, the DHA concentrations ($\leq 10\ \mu M$) used in combination with 5-FU were lower than those widely used in vitro and known to cause peroxidative effects in vivo. *Results*: Whereas the cells showed different sensitivity to the growth-inhibitory action of 5-FU, DHA reduced cell growth independently of p53 cellular status. DHA synergized with 5-FU in reducing colon cancer cell growth. The potentiating effect of DHA was attributable to the enhancement of the proapoptotic effect of 5-FU. DHA markedly increased the inhibitory effect of 5-FU on the expression of the antiapoptotic proteins BCL-2 and BCL-X_L, and induced overexpression of c-MYC which has recently been shown to drive apoptosis and, when overexpressed, to sensitize cancer cells to the action of proapoptotic agents, including 5-FU. *Conclusion*: Our results indicate that DHA strongly increases the antineoplastic effects of low concentrations of 5-FU. Overall, the results suggest that combinations of low doses of the two compounds could represent a chemotherapeutic approach with low toxicity.

Keywords Colon cancer · DHA · 5-FU · Apoptosis

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Introduction

Colorectal cancer is one of the most common malignancies in the Western world [21]. In recent years, it has been increasingly apparent that dietary fatty acids may influence the incidence and growth of colon cancer. In particular, epidemiological studies have shown a reduced incidence of colon cancer among populations consuming a large quantity of n-3 polyunsaturated fatty acids (PUFAs) of marine origin [44, 24, 6]. Moreover, experimental studies have demonstrated that treatment with these fatty acids inhibits carcinogen-induced colon tumorigenesis in rats [45], reduces growth of transplantable colon carcinoma implanted in mice [17, 50], and induces apoptosis in colorectal tumor cells cultured in vitro [35, 36]. We have found that the main components of fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), inhibit the proliferation of colon epithelial cells in patients with sporadic colorectal adenomas at high risk of colon cancer [2, 3]. Accordingly, Huang and colleagues [31] found that supplementation with fish oil concentrate decreases the proliferation of colonic epithelium adjacent to surgically resected cancers in patients at high risk of developing a second neoplasm. On the other hand, we have reported that dietary treatments with EPA and DHA do not modify the homeostasis of normal colon crypts in rats, as shown by the unchanged number of colon mucosa cells and architecture of crypts [14].

The antitumoral activity of n-3 PUFAs, observed also in different cell models, has been related to the modulating effects of these fatty acids on cell

proliferation, differentiation and apoptosis [1, 13–15, 26, 52]. It has been reported that these fatty acids also exert other beneficial effects in neoplastic pathology, such as the ability to inhibit tumor metastasis [32] and angiogenesis [56], to reduce cachexia [51], and to diminish various side effects of different chemotherapeutic agents [30]. In particular, it has been shown that n-3 PUFAs may sensitize several kinds of tumors, including breast cancers [29], sarcomas [33] and leukemias [18], growing in animals to different anticancer drugs. Similarly, treatment of various strains of human tumor cells cultured in vitro (mammary, glioblastoma, lung or leukemic cells) [11, 28, 48, 58] with n-3 PUFA increased their sensitive to doxorubicin and other antineoplastic agents.

In the present study, we sought to determine whether treatment with DHA was able to enhance the weak efficacy of low concentrations of 5-fluorouracil (5-FU) in human colon cancer cells cultured in vitro. Even though this antimetabolite is widely used in therapy for advanced colorectal cancer [34], the response rates to regimens using this agent remain relatively disappointing [34, 41, 53], and considerable side effects have been reported. We also examined several aspects of tumor cell biology after treatment with 5-FU and DHA, including apoptosis induction and cell cycle progression.

In the study reported here, we demonstrated that cotreatment of colon cancer cells lines with DHA and 5-FU produced a greater antineoplastic effect than when the agents were administered individually. Overall, our results suggest an enhancement of the 5-FU proapoptotic effect by DHA through a downregulation of the antiapoptotic proteins BCL-2 and BCL-X_L and an upregulation of c-MYC.

Materials and methods

Cells lines and reagents

The human colon adenocarcinoma cell lines, LS-174, Colo 320 HSR, Colo 205 and HT-29 were obtained from ATCC (Rockville, Md.). LS-174, Colo 205 and Colo 320 HSR cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and HT-29 cells were cultured in MEM supplemented with 1% sodium pyruvate, 2% vitamins, 1% non-essential amino acids and 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. LS-174, Colo 205 and HT-29 cells were serially subcultured by treatment with trypsin–EDTA and seeded twice a week at density of 3×10^5 cells/ml. The same density was used to subculture Colo 320 cells, which were grown in suspension. The experiments were performed using 5% FCS. DHA and 5-FU were purchased from Sigma-Aldrich (Sigma, St Louis, Mo.). DHA was added from an absolute ethanol stock solution and the control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceed 0.5% (v/v).

Growth-inhibitory assay

The cells were seeded in 24-well plates at 3×10^4 cells/well and divided into control (ethanol) and treatment groups (DHA 10–50 μ*M*, 5-FU 0.1–7.5 μ*M*, and DHA plus 5-FU in combination). The medium was removed on the third day and fresh medium along with various concentrations of DHA and/or 5-FU were added to the cultures. After 6 days of exposure, the cells were harvested, stained with trypan blue and counted under an inverted-phase microscope using a Burker chamber (quadruplicate hemocytometer counts of triplicate cultures were performed).

Clonogenic assay

Clonogenic assay was performed as described by Conneally et al. [20]. Briefly, the cells (3×10^4 ml^{-1}) were suspended in a medium containing 0.8% methylcellulose (Methocult H4100, Stem Cell technologies), 5% FCS, 2 m*M* L-glutamine, and vehicle alone (ethanol) or with drugs (DHA, 5-FU, or their combination) and then plated in 35 mm Petri dishes. Colonies (aggregation of 30 or more cells) were scored in situ after 11 days of incubation at 37°C in a humidified atmosphere comprising 5% CO_2 in air.

Detection of apoptosis

After treatment, the proportion of apoptotic cells was determined using the immunocytochemical TUNEL assay, as previously described [40]. Briefly, cytocentrifuge cell preparations were fixed with acetone and incubated for 5 min. Then, 2.5 U terminal deoxynucleotidyl transferase (TdT) and 100 pmol biotin–dUTP were added followed by incubation for 1 h at 37°C. Thereafter, the cells were incubated with the streptavidin–biotin–peroxidase complex for 30 min at room temperature. Peroxidase activity was detected with 3,3′-diaminobenzidine tetrahydrochloride. At least 100 cells per microscopic field were evaluated. To confirm the results of the TUNEL analysis, apoptosis was evaluated morphologically by staining the cells with acridine orange (100 μg/ml) and analyzing them by fluorescence microscopy under high-power magnification (×400), as previously described [16].

Cell cycle analysis

Cell cycle distribution was determined by flow cytometry, as previously described [23]. Briefly, after treatment, the cells were dissociated using trypsin–EDTA, pelleted and resuspended in 1 ml PBS. While vortexing, 5 ml 70% ethanol was added dropwise. The cells were incubated at 4°C for 30 min and then centrifuged at 2,500 *g* for 10 min. The cells were treated with 1 mg/ml RNase

for 30 min in the dark at room temperature. Propidium iodide was added to a final concentration of 50 μg/ml. The stained nuclei were analyzed on an Epics Profile flow cytometer (Coulter Electronics, Hialeah, Fl.) with an argon laser (Omnicrome 500, 15 mW, excitation wavelength 488 nm). Data were collected, stored and analyzed using Multicycle software (Phoenix, S. Diego, Calif.).

Western blot analysis

Cell extracts were prepared by lysing the cells (1×10^7) in ice-cold lysis buffer (1 m*M* $MgCl_2$, 350 m*M* NaCl, 20 m*M* Hepes, 0.5 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* $Na_4P_2O_4$, 1 m*M* PMSF, 1 m*M* aprotinin, 1.5 m*M* leupeptin, 20% glycerol, 1% NP-40) as previously described [23]. The protein content was determined by Bradford method using the Biorad assay (Hercules, Calif.) [8]. Equal amounts of proteins (50 μg) were separated on a 12% SDS polyacrylamide gel and electroblotted to a nitrocellulose membrane. The membrane was blocked overnight at 4°C in 5% dried milk (w/v) in PBS plus 0.05% Tween 20 and then incubated with specific monoclonal antibodies to BCL-2, BCL-X_L, and c-MYC proteins (Santa Cruz Laboratories, Santa Cruz, Calif.). Following incubation with anti-mouse secondary antibody (Amersham, Pharmacia Biotech Italia, Milan, Italy), the immunocomplexes were visualized using the enhanced chemiluminescence detection system (ECL, Amersham) and quantitated by densitometric scanning.

Data analysis

The results are expressed as the means ± SE. The data were analyzed using one-way analysis of variance (ANOVA). Post hoc comparison of means was made using Fisher's test (significance $P<0.05$). Differences were analyzed using Minitab Software (Minitab, State College, Pa.). The combined effects of 5-FU and DHA (combination indices) were calculated using the formula %AB/%A × %B, where A and B are the effects of each individual agent and AB is the effect of the combination. When the ratio (combination index) is 1 the effect is considered additive; when the combination index is significantly greater than or less than 1, the effect is considered subadditive (negative synergism) or supraadditive (positive synergism), respectively [22, 42]. Statistical significance values of the combination indices were compared with the additive combination index of 1 by one-sided Student's *t*-test.

Results

The effects of increasing concentrations of 5-FU and DHA on growth of colon cancer cells (LS-174, Colo 320 HSR, Colo 205 and HT-29 cells) were assessed by cell counting after a 6-day treatment (Table 1 and Fig. 1). Since it has been shown, both in vitro and in vivo, that the efficacy of 5-FU is often related to the p53 status of human colon cancer cells [10], we analyzed the effects of 5-FU and DHA on cell growth both in p53-wildtype and p53-mutant colon cancer cells. Among the cells tested, LS-174 and Colo 320 HSR are known to be p53-wildtype, whereas HT-29 and Colo 205 are reported to be p53-mutant, carrying different p53 mutations [38]. DHA inhibited growth of all the cells studied (Table 1) and its action was not related to p53 status of the cells, as shown by the DHA concentrations inhibiting cell growth by 20% and 50% ($IC_{20(DHA)}$ and $IC_{50(DHA)}$). $IC_{20(DHA)}$ ranged from 2.5 to 10.1 μ*M* and $IC_{50(DHA)}$ from 5.5 to 37.2 μ*M* in the different cells, but independently from the p53 status. Also 5-FU inhibited growth of all the cells analyzed (Fig. 1), but higher values of $IC_{50(5\text{-}FU)}$ were obtained for the p53-mutant cells (Table 1) as compared to those found for the p53-wildtype cells, confirming that p53-mutant cells are more resistant to the growth-inhibitory effects of 5-FU. We report also the $IC_{20(5\text{-}FU)}$ values, which correspond to the concentrations used in all the combination experiments. We excluded higher concentrations of 5-FU (from 5- to 50-fold higher than the concentrations used in the present work), currently found in the plasma of patients treated by continuous infusion of the drug, which are known to cause marked adverse health effects in patients [27].

Figure 1 shows also that the effects of increasing concentrations of 5-FU on the growth of colon cancer cells were markedly enhanced when this antimetabolite was administered in combination with DHA (given at its IC_{20}). The $IC_{20(DHA)}$ was chosen for the following combination experiments, avoiding higher, more effective concentrations widely used in in vitro experiments, since it has been shown that high concentrations of PUFAs cause deep peroxidative effects on cell membranes when administered in vivo to animals or human subjects [39]. The analysis of the interaction between the inhibitory effects of DHA and 5-FU (both given at their IC_{20}) on colon cancer cell growth revealed (Table 2) a combination index <1 for all the strains of cells

Table 1 Inhibitory effect of DHA and 5-FU on colon cancer cell growth. Cells were exposed to DHA or 5-FU for 6 days. Cell growth was evaluated counting the cells under an inverted-phase microscope. Data are the means ± SE obtained from at least four counts of triplicate cultures

	p53-wildtype cells		p53-mutant cells	
	LS-174	Colo 320 HSR	Colo 205	HT-29
DHA				
IC_{20} (μ*M*)	10.12 ± 1.68^a	4.09 ± 0.75^b	2.50 ± 0.29^c	10.05 ± 1.27^a
IC_{50} (μ*M*)	37.24 ± 2.89^a	13.26 ± 1.78^b	5.51 ± 0.65^c	30.23 ± 0.42^d
5-FU				
IC_{20} (μ*M*)	0.10 ± 0.01^a	0.33 ± 0.02^b	0.62 ± 0.04^c	1.02 ± 0.09^d
IC_{50} (μ*M*)	0.43 ± 0.03^a	0.93 ± 0.05^b	1.55 ± 0.08^c	5.50 ± 0.47^d

[a–d]Values sharing different letters are significantly different ($P<0.05$, one way ANOVA, followed by Fisher's test)

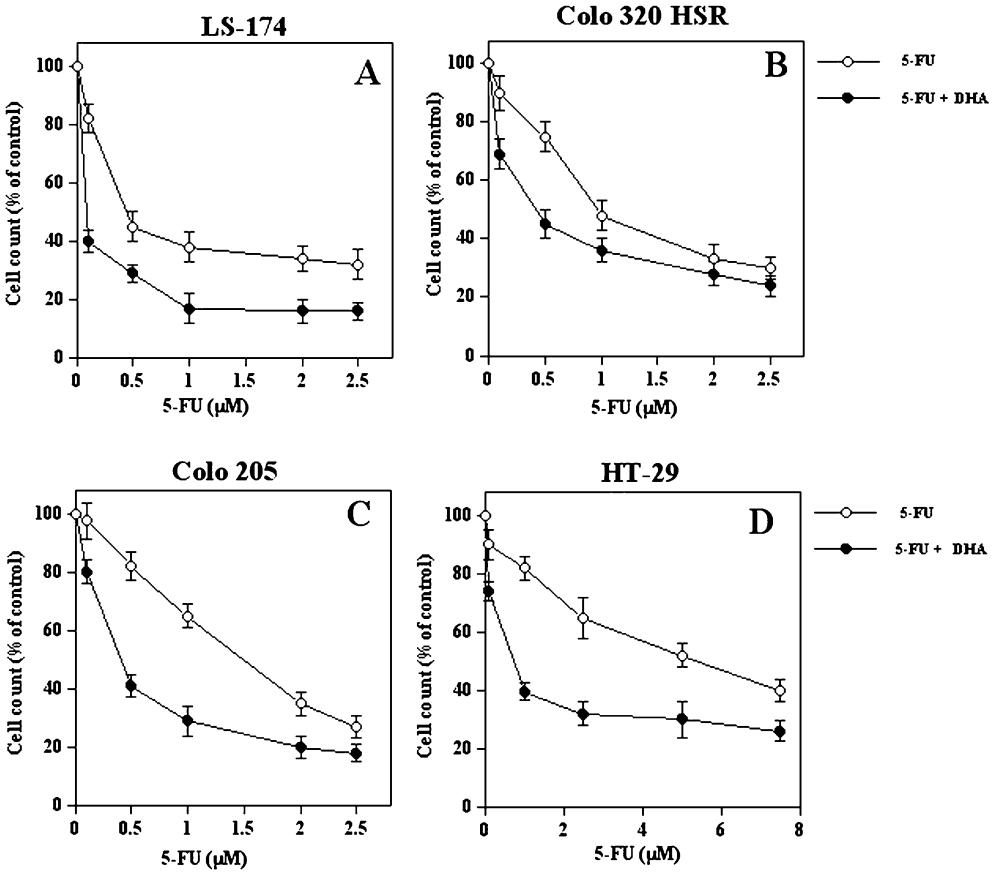


Fig. 1a–d Effect of increasing concentrations of 5-FU in the absence or presence of DHA on colon cancer cell growth. Cells were exposed to 5-FU and DHA for 6 days. DHA was used at its IC_{20}. The percentages of cells were determined by counting them under an inverted-phase microscope. Data points represent the means ± SE of at least four counts of triplicate cultures

analyzed, indicating a positive synergistic effect between the two compounds. Similar synergistic growth-inhibitory effects were observed also after shorter treatment times (24, 48 and 96 h) with the two substances administered at their IC_{20} (data not shown), but longer periods of treatment (6 days) are generally preferred to study the effects of chemotherapeutic agents on neoplastic cells, easily subject to drug resistance [54]. Since such a strong potentiation by DHA of the growth-inhibitory effect of 5-FU was observed in all the cells tested, independent of their p53 status, we carried out all the remaining experiments using only one of the cell lines (LS-174).

Table 2 Analysis of the interaction between the inhibitory effect of DHA in combination with 5-FU on colon cancer cell growth. The data utilized for the analysis are those obtained combining the two substances at their IC_{20} and reported also in Fig. 1. The combination index was calculated using the formula %AB/%A × %B, as described under "Materials and methods". The values presented are the means ± SE of quadruplicate determinations

Cell line	Combination index	*P* value[a]
LS-174	0.167 ± 0.012	< 0.01
Colo 320 HSR	0.112 ± 0.014	< 0.05
HT-29	0.179 ± 0.018	< 0.01
Colo 205	0.154 ± 0.014	< 0.01

[a]Compared with the additive combination index of 1 by one-sided Student's *t*-test.

In these cells we confirmed the results obtained by cell count also investigating the inhibitory effect exerted by the 5-FU/DHA combination on colony formation by clonogenic assay (Fig. 2), which is considered a very reliable method for assessing the efficacy of chemotherapeutic agents in vitro [9]. The 5-FU/DHA combination at low concentrations (10 μ*M* DHA or 0.1 μ*M* 5-FU, IC_{20} values obtained for LS-174) decreased the surviving fraction much more efficiently than each compound given alone (5-FU/DHA combination 69% reduction; DHA and 5-FU 13% and 25% reduction, respectively), indicating a clear positive synergistic effect (combination index 0.212 ± 0.02, $P < 0.001$).

We next examined whether the growth-inhibitory effects of the 5-FU/DHA combination could be ascribable to its ability to induce apoptosis. Using the TUNEL assay we observed that a 24-h treatment with 10 μ*M* DHA or 0.1 μ*M* 5-FU given separately induced apoptosis in LS-174 cells with a similar efficacy (about 3.5-fold increase in the percentage of apoptotic cells; Fig. 3a). A more powerful effect was noticed when the two compounds were administered in combination (12-fold increase). The positive synergistic effect (combination index 0.0090 ± 0.0001, $P < 0.001$) of DHA on the proapoptotic capacity of 5-FU was also confirmed when apoptosis was evaluated morphologically in cells stained with acridine orange and ethidium bromide (Fig. 3b). We limited the observation on apoptosis to the first 24 h since when the morphological analysis was performed

following 48 or 72 h of treatment, a substantial proportion of cells became stained with the membrane-impermeant dye ethidium bromide, indicating postapoptotic alterations of necrosis (data not shown).

The marked induction of apoptosis after 24 h of 5-FU/DHA treatment was further confirmed by the analysis of cell cycle progression in LS-174 cells (Fig. 4), and was indicated by an increase of the sub-G_1 phase cellular pool. On the other hand, the cell cycle distributions of LS-174 cells were only slightly modified by DHA and 5-FU, added alone or in combination, with the G_0/G_1 and S phases decreasing and the G_2/M phase increasing after both DHA and 5-FU treatment. However, a marked decrease in the G_0G_1/G_2M ratio was observed in the presence of 5-FU or DHA (30.0% and 31.5% decrease, respectively), and the reduction in the ratio became much more conspicuous (58%) when the two compounds were given in combination. These findings suggest that the two substances, particularly when administered in combination, markedly induce the progression of cells from G_1 to G_2/M phase, from which they may undergo apoptosis.

We next analyzed the alteration caused by 5-FU and DHA treatments in the expression of several proteins involved in the regulation of apoptosis. First we examined two proteins of the BCL-2 family, BCL-2 and BCL-X_L, known to exert antiapoptotic effects (Fig. 5). DHA (10 μM) and 5-FU (0.1 μM) inhibited the expression of BCL-2 by 33.1 ± 1.7 and 17.0 ± 0.9%, respectively, and by 54.1 ± 4.3% in combination, indicating an additive effect (combination index 1.04 ± 0.02, $P = 0.05$). Moreover, DHA and 5-FU inhibited BCL-X_L expression by 33.5 ± 2.7% and 28.1 ± 2.2%, respectively. In this case, when the compounds were given in combination, a positive synergistic effect was noticed (80.1 ± 5.1% inhibition, combination index 0.087 ± 0.001, $P < 0.001$).

Furthermore, since the ability of c-MYC, a crucial regulator of cell proliferation, to drive apoptosis in normal and neoplastic cells has recently also been shown, and since c-MYC overexpression has been shown to sensitizes colon cancer cells to apoptosis [4], we analyzed the change in the expression of this protein after DHA and 5-FU treatments, alone or in combination in LS-174 cells (Fig. 6). Whereas DHA was able to increase the expression of c-MYC (52 ± 5% increase), 5-FU did not significantly altered it. Moreover, the effect of DHA was not modified by the simultaneous addition of 5-FU. Similar modifications in the expression of BCL-2 and BCL-X_L and c-MYC proteins were observed in p53-mutant HT-29 cells treated with 5-FU and DHA at their IC_{20} (data not shown).

These data suggest that both DHA and 5-FU are able to inhibit the expression of anti-apoptotic proteins BCL-2 and BCL-X_L, but only DHA has a remarkable effect on the proapoptotic protein c-MYC.

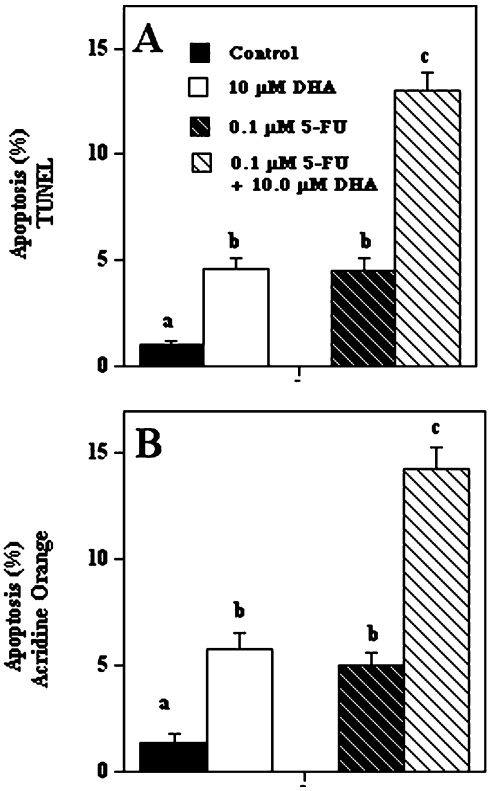


Fig. 3a, b Induction of apoptosis by 5-FU and DHA, alone or in combination, in LS-174 cells. Cells were exposed to 5-FU and DHA at their IC_{20} for 24 h. Apoptosis was evaluated by immunocytochemical TUNEL assay (**a**) or by fluorescence microscopy after staining the cells with acridine orange (**b**). The results presented are the means ± SE of at least four experiments. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA, followed by Fisher's test)

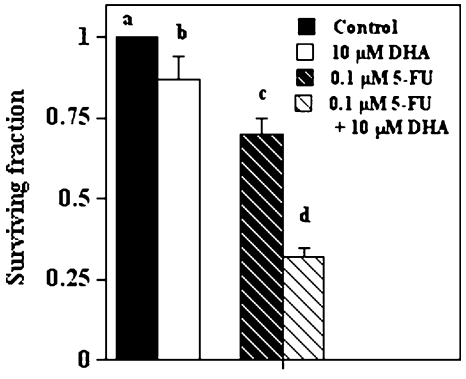
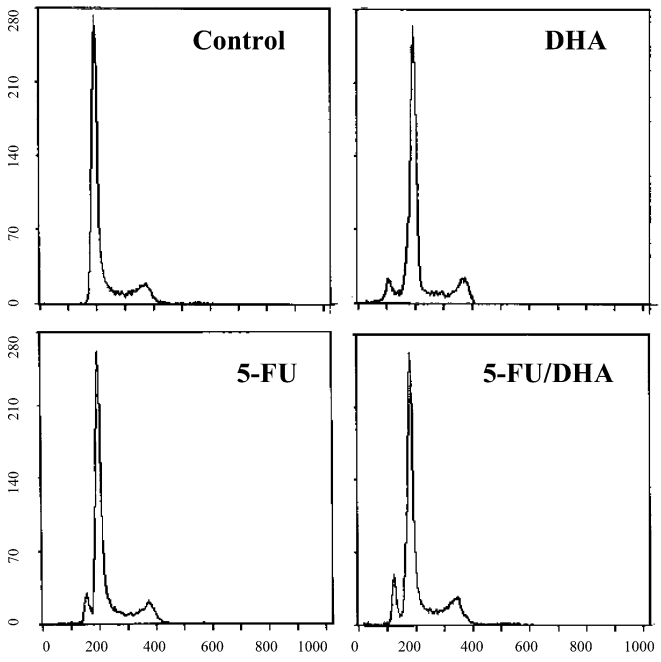


Fig. 2 Effect of 5-FU and DHA alone or in combination on colony formation in LS-174 cells. The colonies were measured by clonogenic assay, counting them 11 days after plating. 5-FU and DHA were used at their IC_{20}. The results presented are the means ± SE of four experiments. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA, followed by Fisher's test)

Discussion

It has recently been shown that colon cancer cells, both cultured in vitro or growing in experimental animals, are sensitive to the growth-inhibitory action of n-3 PUFA



	Control	DHA	5-FU	5FU/DHA
Sub G1	1.8 ± 0.1^a	7.4 ± 0.3^b	7.8 ± 0.7^b	11.2 ± 0.5^c
G0/G1	66.0 ± 2.7	63.6 ± 3.1	63.0 ± 2.4	61.3 ± 1.7
S	25.2 ± 1.6^a	21.1 ± 2.0^a	22.3 ± 1.5^a	16.3 ± 0.9^b
G2/M	5.8 ± 0.5^a	7.4 ± 0.3^b	7.4 ± 0.5^b	11.5 ± 1.0^c

Fig. 4 Cell cycle distribution of LS-174 after DHA or 5-FU treatments or 5-FU/DHA cotreatment. The compounds were used at their IC_{20}. One representative of four similar flow cytometric profiles is shown for every condition. In each panel the percentage of phase distribution is also shown. DNA cell cycle analysis was performed by propidium iodide labeling as described under "Materials and methods"

[7, 12]. We have found that EPA and DHA, the major n-3 PUFAs present in fish oils, inhibit the proliferation of colon mucosa in patients at high risk of colon cancer [2, 3]. Moreover, it has recently been reported that n-3 PUFAs enhance susceptibility to the action of various anticancer drugs in different tumor cells growing in animals or cultured in vitro, including mammary [28, 29], lung [58], glioblastoma [48], leukemic [11] and sarcomatous cells [33]. The objective of our study was to test the ability of DHA to enhance the growth-inhibitory action of the antineoplastic drug 5-FU on colon cancer cells and to verify whether this effect can be exerted through the observed capacity of DHA to modulate apoptotic process [1, 13, 14, 16, 26, 52]. To do this, we treated different colon cell lines with combinations of low concentrations of 5-FU and DHA (at their IC_{20}). The use of these low doses given in combination was related to our hypothesis that the weak efficacy of low 5-FU concentrations may be markedly enhanced by concomitant treatment with concentrations of DHA easily achievable in vivo by dietary supplementation.

Since it is known that the efficacy of 5-FU is related to p53 cell status [55], we investigated the growth-inhibitory effect of 5-FU and DHA both in p53-wildtype (LS-174 and Colo 320 HSR) and in p53-mutant (HT-29 and Colo 205) human tumor cell lines. By cell count experiments after 6 days of treatment we confirmed that the growth-inhibitory effect of 5-FU was related to the presence of a functionally active p53 protein, since we found $IC_{50(5\text{-}FU)}$ values above 1.5 μM for the mutant cells (Colo 205 and HT-29) and in the submicromolar range for the wildtype cells (LS-174 and Colo 320 HSR). On the other hand, unlike 5-FU, the growth-inhibitory effect of DHA was not related to p53 status, as showed by the calculated $IC_{50(DHA)}$ values which were in some cases even higher for the p53-wildtype than for the p53-mutant cells. Moreover, in all the cells the concomitant addition of 5-FU and DHA at low concentrations (IC_{20}) inhibited cell growth in a synergistic manner, as shown by the combination index always being significantly lower than 1.0.

Since the synergistic effects of the two compounds on the inhibition of cell growth were similar in all the strains of colon cells tested, we limited our further investigations to one strain of colon cells, the LS-174 cells. In these cells the effect was also confirmed by evaluating the cell colon forming efficiency, an in vitro assay widely reported to be more predictive of the growth inhibitory effects exerted in vivo by the chemotherapeutic agents [9].

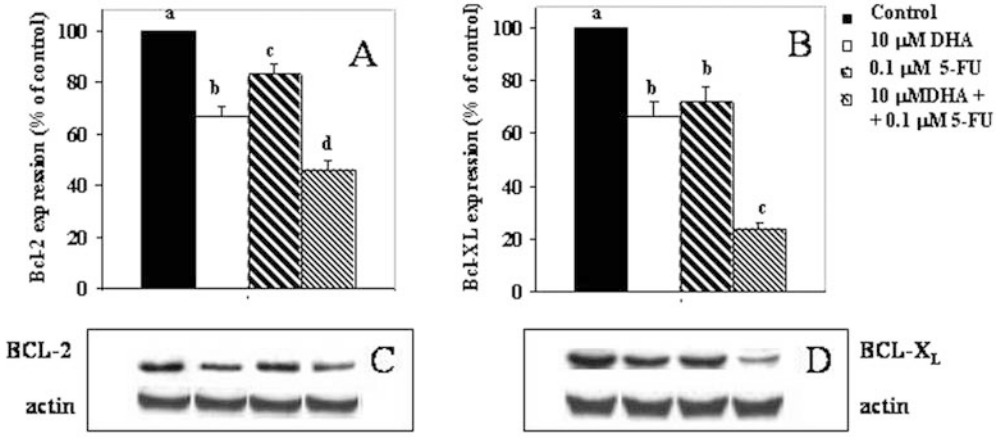


Fig. 5a–d Effect of 5-FU and DHA alone and in combination on BCL-2 and BCL-X_L expression in LS-174 cells. The compounds were used at their IC_{20}. **a** BCL-2 expression as percent of control; **b** BCL-X_L expression as percent of control. The results presented are the means ± SE of at least four experiments. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA, followed by Fisher's test). One representative of four similar Western blot analyses is shown for BCL-2 (**c**) and BCL-X_L (**d**)

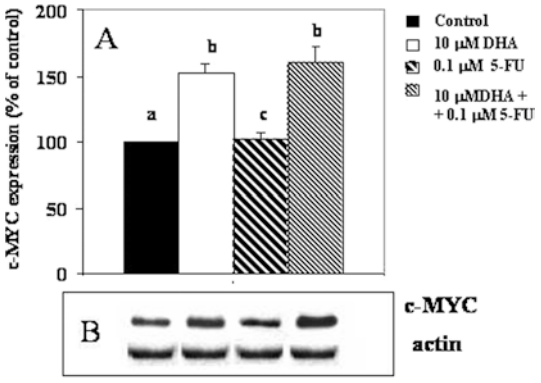


Fig. 6a, b Effect of 5-FU and DHA alone and in combination on c-MYC expression in LS-174 cells. The compounds were used at their IC_{20}. **a** c-MYC expression as percent of control. The results presented are the means ± SE of five experiments. Values with different letters are significantly different ($P < 0.05$, one-way ANOVA, followed by Fisher's test). **b** One representative of five similar western blot analyses is shown for c-MYC

Confirming previous observations [19, 37], showing a powerful apoptogenic effect of DHA acid in colon cancer cells, in the present work, both using the TUNEL assay and analyzing the morphological apoptotic features in acridine orange-stained cells, we demonstrated that DHA is able to induce apoptosis in LS-174 cells as early as at 24 h, even if added at low concentrations (IC_{20}). Moreover, in agreement with the reported proapoptotic effect of 5-FU [47, 57], the low concentrations (IC_{20}) of 5-FU used in the present study were also able to induce apoptosis in LS-174 cells. DHA administered concomitantly with 5-FU (both at their IC_{20}) markedly potentiated apoptosis induced by this antimetabolite showing a clear positive synergistic effect. We suggest that the enhancement of 5-FU-driven apoptosis by DHA in colon cancer cells may be at the basis of the potentiating effect exerted by this fatty acid on the cell growth-inhibitory effect of 5-FU.

Cytofluorimetric cell cycle distribution analysis in LS-174 cells after 24 h of treatment revealed an increase in the sub-G_1 cellular (apoptotic) pool in the presence of DHA and/or 5-FU, alone or in combination, confirming the proapoptotic effect of these agents. Moreover, the cell cycle distribution also showed a marked decrease in the G_0G_1/G_2M ratio, which may suggest arrest of cells in the G_2/M compartment before undergoing apoptosis.

Since the growth-inhibitory effect of DHA and 5-FU, alone or in combination, appeared to be related to their ability to induce apoptosis, we evaluated the alterations induced by 5-FU and DHA in the expression of several proteins of the BCL-2 family, known regulators of apoptosis [46]. Apoptosis induction by DHA and 5-FU was related to the inhibition of the antiapoptotic proteins BCL-2 and BCL-X_L. Confirming previous results [19], we found that DHA was able to inhibit the expression of BCL-2. Moreover, it also reduced the expression of BCL-X_L. The levels of these antiapoptotic proteins were particularly reduced when DHA acted in concert with 5-FU, indicating an additive effect for BCL-2 expression and a clear positive synergistic effect for BCL-X_L expression. We suggest that this potentiating action may partially explain the synergistic effects exerted by DHA on both the 5-FU induction of the apoptotic process and inhibition of human colon cancer cell growth. Another possible mechanism underlying the remarkable growth-inhibitory and proapoptotic effect of the 5-FU/DHA combination is the induction of the expression of c-MYC, caused only by DHA in colon cancer cells. It has recently been reported that elevated c-MYC expression sensitizes colon cancer cells to apoptosis induced by different stimuli, including low serum conditions, hypoxia and deprivation of specific growth factors [5, 25, 49]. Moreover, it has been shown that elevation of c-MYC in colon cancer cells produced by introducing a c-myc expression vector increases the sensitivity LoVo colon cancer cells to 5-FU-induced apoptosis [4]. On this basis, it is possible to suggest that the upregulation of this protein may partially explain the proapoptotic effect of DHA. On the other hand, in cells treated concomitantly with DHA and 5-FU, c-MYC expression may render the cells more sensitive to the proapoptotic action of 5-FU, providing a further possible explanation for the synergistic action of DHA on the proapoptotic and growth-inhibitory effect of 5-FU. Since it has been recently shown that c-MYC is able to induce both p53-dependent and p53-independent pathways of apoptosis [43], this mechanism could act irrespective of the p53 status of the cells.

In conclusion, the synergistic effects exhibited by low concentrations of DHA in enhancing the induction of apoptosis and the reduction in colonic cancer cell growth by low concentrations of 5-FU, suggest that combinations of low doses of the two compounds could represent a chemotherapeutic approach with low toxicity.

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