Acriflavine enhances the antitumor activity of the chemotherapeutic drug 5-fluorouracil in colorectal cancer cells

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Abstract. 5-Fluorouracil (5-FU)-based chemotherapy improves the overall survival rates of patients with colorectal cancer (CRC). However, only a small proportion of patients respond to 5-FU when used as a single agent. The aim of the present study was to investigate whether the anticancer property of 5-FU is potentiated by combination treatment with acriflavine (ACF) in CRC cells. Additionally, the potential underlying molecular mechanisms of the cytotoxic effect of ACF were determined. The cytotoxic effects of ACF, 5-FU and irinotecan on different CRC cell lines with different p53 status were investigated using an MTT assay. SW480 cells that express a mutated form of p53 and two other CRC cell lines were used, HCT116 and LS174T, with wild-type p53. To determine the effect of ACF on the sensitivity of cells to 5-FU, cells were co-treated with the 30% maximal inhibitory concentration (IC_{30}) of ACF and various concentrations of 5-FU, or pretreated with the IC_{30} of ACF and various concentrations of 5-FU. To assess the mechanism of action of ACF, cells were treated with IC_{30} values of the compound and then the reverse transcription-quantitative polymerase chain reaction was used to evaluate mRNA levels of hypoxia-inducible factor-1α (HIF-1α) and topoisomerase 2. Results indicate that pretreatment with ACF markedly sensitized CRC cells to the cytotoxic effects of 5-FU, whereas simultaneous treatment with ACF and 5-FU were not able to alter the resistance of CRC cells to 5-FU. In comparison with irinotecan, ACF was a more potent agent for enhancing the antitumor activity of 5-FU. ACF did not alter the mRNA levels of either HIF-1α or topoisomerase 2. The results of the present study reveal for the first time that pretreatment of CRC cells with ACF markedly increases the cytotoxic effects of 5-FU, regardless of the p53 status of cells.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the second leading cause of cancer-associated mortality globally (1,2). The major CRC prognostic factor is the stage at diagnosis. Early diagnosis of patients with CRC allows a 5-year survival rate of 90%, but <10% when advanced metastases occur (3). Between 20 and 25% of patients diagnosed with the disease already have metastases to other organs, and between 50 and 60% of the remaining patients will develop metastases (4,5).

The main treatment for CRC is surgery. However, in patients with advanced CRC, surgery is not always able to prevent progression of the disease. Therefore, chemotherapy is used complementarily to decrease the risk of local recurrence (5,6). In total, ~50% of patients with CRC are candidates to receive chemotherapy (5,6). 5-Fluorouracil (5-FU) has been the first-line and gold standard of chemotherapy for the treatment of advanced CRC. 5-FU, a pyrimidine antagonist, is converted intracellularly into active metabolites that exert antitumor effects through the inhibition of thymidylate synthase and disruption of RNA and DNA synthesis (7).

It is clear that 5-FU-based chemotherapy decreases tumor recurrence and improves the overall survival rates of patients with advanced CRC. However, only between 10 and 15% of patients respond to 5-FU as a single first-line treatment as drug resistance limits the effectiveness of monotherapy (8). To enhance the antitumor activity of 5-FU and overcome its clinical resistance, this drug has been used in combination with other cytotoxic agents. Different combinations of these agents with 5-FU as the principal drug have been used to develop a variety of chemotherapy protocols to treat patients with advanced CRC. These modern chemotherapy regimens, including 5-FU + lucoverin + irinotecan, 5-FU + oxaliplatin and capecetabine (a 5-FU prodrug) + oxaliplatin with or

without monoclonal antibodies, have improved the response rate and outcome in patients with advanced CRC (6,9-11). Despite these substantial advances, the long-term survival of patients with metastatic CRC has not been achieved (6). Therefore, the design of novel chemotherapy protocols using more active drugs with fewer side effects is urgently required.

Acriflavine (ACF), a naturally occurring compound, is a mixture of 3,6-diamino-10-methylacridinium chloride (trypaflavin) and 3,6-diaminoacridine (proflavine) and has a history of clinical use (12). ACF is a US Food and Drug Administration-approved drug that has been administered topically or systemically for the treatment of microbial infections (12). The median lethal dose (LD_{50}) of ACF in humans is unclear, but the LD_{50} of ACF in mice is 30 mg/kg (13). It has been demonstrated that ACF exhibits antitumor activity in several types of cancer, including breast cancer, osteosarcoma and hepatocellular carcinoma (14-16). It has also been demonstrated previously that ACF limits tumor growth and progression in mouse models of colorectal cancer through inhibition of hypoxia-inducible factor (HIF) (17). Importantly, long-term administration of ACF to patients as an antiviral agent has not revealed any major side effects (18).

Several molecular mechanisms have been proposed for the anticancer property of ACF. Studies by Shay *et al* (17) and Hassan *et al* (19) proposed that cytotoxic property of ACF in CRC cells may be associated with inhibition of topoisomerase 2 and HIF-1α activity. However, the exact molecular mechanism of action of ACF against CRC remains to be determined (19). To the best of our knowledge, it has not been investigated previously whether ACF is able to act through the alteration of mRNA expression of these two important proteins in CRC cells.

The aim of the present study was to investigate whether it was possible to potentiate the anticancer property of 5-FU when combined with ACF in CRC cells. If this combination protocol significantly enhanced the efficacy of 5-FU based chemotherapy, it may be a basis for the development of other preclinical and clinical studies to design new chemotherapy regimens using ACF for those patients with advanced CRC who are 5-FU-resistant. In addition, the effect of ACF on the mRNA expression level of topoisomerase 2 and HIF-1α was evaluated as a potential molecular mechanism underlying the cytotoxic effect of this drug on CRC cells.

Materials and methods

Chemicals and reagents. ACF, 5-FU, irinotecan and MTT were purchased from Sigma; Merck KGaA (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was from Merck KGaA. Other reagents used for cell culture were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Reagents were prepared and stored according to the manufacturers' protocol.

Cell lines and cell culture. The human colon cancer cell lines SW480, HCT116 and LS174T were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were cultured in either RPMI-1640 medium (SW480) or Dulbecco's modified Eagle's medium (DMEM; HCT116 and LS174T) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Drug cytotoxicity assay. An MTT assay was used to determine the cytotoxic effect of ACF, 5-FU and irinotecan, a standard chemotherapy drug routinely used with 5-FU, on CRC cell lines, as described previously (20). The optimum number of cells/well for 72 h of incubation was first determined. CRC cells were seeded in 96-well plates at density of $8x10^3$ cells/well in 100 μl DMEM or RPMI medium. At 1 day after seeding, ACF (0.07-5 μM), 5-FU (0.125-128 μM) and irinotecan (2.5-80 μM) were added at the specified concentration and incubated at 37˚C for 24, 48 or 72 h. The medium of untreated control cells was replenished with medium without drugs. Following drug treatment, 20 μl MTT reagent was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h at 37˚C in a humidified atmosphere containing 5% CO_2. The medium was then aspirated and crystals were dissolved in 150 μl DMSO/well. The optical density at 570 nm (OD_{570}) was determined using a microtiter plate reader. After 72 h, the confluency of untreated control was between 80 and 90%. Cell viability was calculated using the following formula: Cell viability=OD_{570} (sample)/OD_{570} (control) x100.

Determination of half-maximal inhibitory concentration (IC_{50}) and 30% maximal inhibitory concentration (IC_{30}). The IC_{50} and IC_{30} values associated with the cytotoxic effects of the drugs were calculated using GraphPad Prism software (version 5.00; GraphPad Software, San Diego, CA, USA) using non-linear regression model and dose-response equations.

Drug co-treatment protocol. The stock solutions of ACF and 5-FU were prepared and diluted in cell culture medium. Cells were treated with different concentrations of 5-FU (0.5, 1, 2, 4, 8, 16, 32 and 64 μM) or ACF (0.07, 015, 0.31, 0.62, 1.25, 2.5 and 5 μM) for 72 h. The IC_{50} values for the cytotoxic effects of either 5-FU or ACF were subsequently calculated. To evaluate the effect of ACF on the sensitivity of cells to 5-FU, cells were simultaneously treated with a low cytotoxic concentration (IC_{30}) of ACF and different concentrations of 5-FU for 72 h. The cell viability and the IC_{50} value of 5-FU in the co-treatment protocol was compared with the IC_{50} value of 5-FU when used for 72 h as a single drug.

Drug pretreatment protocol. CRC cells were treated with different concentrations of ACF (0.15, 0.31, 0.62, 1.25, 2.5 and 5 μM) or 5-FU (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 μM) for 24 and 48 h, respectively. IC_{50} values for the cytotoxic effects of 5-FU and IC_{30} values of ACF were calculated. To evaluate the effect of ACF on the sensitivity of cells to 5-FU, cells were pretreated with the IC_{30} of ACF for 24 h, then the medium was aspirated and replenished with a medium containing 5-FU (at concentrations between 0.125 and 128 μM) for another 48 h. In the same protocol, the effect of irinotecan (IC_{30} concentration table III), a standard chemotherapy drug, on the sensitivity of CRC cells to 5-FU was also assessed and compared with the results of the ACF pretreatment experiment. The overall time for drug treatment in each protocol was 72 h.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. CRC cells (250,000 cells/well) were cultured in 6-well plates and treated with the IC_{30} of ACF for 24 h. Following treatment, total

Table I. Primer sequences used for quantitative polymerase chain reaction analysis.

Gene	Forward primer	Reverse primer
β-actin	5'-GCCTTTGCCGATCCGC-3'	5'-GCCGTAGCCGTTGTCG-3'
HIF-1α	5'-AGGAAATGAGAGAAATGCTTA-3'	5'-GGTTGGTTACTGTTGGTAT-3'
Topoisomerase 2	5'-ATGTATCACCTTTCAGCCT-3'	5'-TTCATCCAACTTGTCCTTC-3'

HIF-1α, hypoxia-inducible factor-1α.

RNA was extracted using an miRNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. The quality and quantity of RNA were determined using agarose gel electrophoresis and a NanoDrop 1000 instrument (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA), respectively. A 1 μg amount of total RNA was used for cDNA synthesis using a PrimeScript™ First-Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). qPCR assays for the quantitative determination of HIF-1α, topoisomerase 2 and β-actin (internal control) were performed in duplicate using a Corbett RotorGene RG-6000 instrument (Qiagen, Inc.). Primer sequences are presented in Table I. Amplifications were performed in 25 μl mixtures containing 2 μl cDNA, 1 μl 10 μM solutions of each of the forward and reverse primers, along with 12.5 μl SYBR Green PCR master mix (SYBR Premix Ex Taq™, Takara Bio). The thermocycling conditions consisted of initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, annealing at 59˚C (β-actin), 52˚C (HIF-1α) and 56˚C (Topoisomerase 2) for 30 sec and extension at 60˚C for 30 sec. The relative amount of mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (21) and normalized to the level of β-actin.

Statistical analysis. Results are expressed as the mean ± standard deviation. Differences between IC_{50} values of three or more groups were determined using a Kruskal-Wallis test and Dunn's post hoc test. A Mann-Whitney U test was performed on experiments with two groups. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS software (version 12.0; SPSS, Inc., Chicago, IL, USA).

Results

Effect of ACF and 5-FU co-treatment on the sensitivity of CRC cells to 5-FU. To assess the cytotoxic effects of ACF or 5-FU, CRC cells (SW480, HCT116 and LS174T) were treated with a graded concentration of drugs for 72 h and cell viability was determined using an MTT assay. The IC_{50} values of ACF for SW480, HCT116 and LS174T cells were 0.75±0.10, 0.57±0.22 and 0.36±0.05 μM, respectively. ACF caused inhibitory effects on the cell growth in a dose-dependent manner (Fig. 1). The same pattern was also obtained for 5-FU (data not shown). The IC_{30} values of ACF for SW480, HCT116 and LS174T cells were 0.36±0.07, 0.29±0.14 and 0.20±0.04 μM, respectively. The sensitivity of CRC cells against ACF and 5-FU was determined by calculation of the IC_{50} values as presented in Table II.

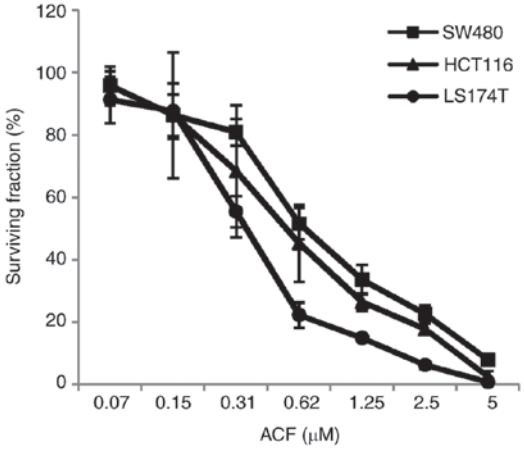

Figure 1. Sensitivity of colorectal cancer cell lines to ACF. Cell viability was determined using an MTT assay following treatment of cells with graded concentrations of ACF for 72 h. Results are the mean ± standard deviation of three experiments, each performed in triplicate. ACF, acriflavine.

SW480 cells exhibited the highest resistance to ACF and 5-FU in comparison with the other two cell lines. To determine the effect of ACF on the sensitivity of cells against 5-FU, cells were simultaneously treated with the IC_{30}, the low cytotoxic concentration, of ACF and different concentrations of 5-FU for 72 h. As indicated in Table II, the results indicated that the co-treatment protocol was not able to significantly alter the IC_{50} value of 5-FU on CRC cells. Therefore, an alternative treatment protocol was designed (pretreatment protocol).

Effects of ACF pretreatment on the sensitivity of CRC cells to 5-FU. To investigate the effects of ACF pretreatment on 5-FU cytotoxicity, CRC cells were pretreated with the IC_{30} of ACF for 24 h, and the cells were incubated with various concentrations of 5-FU and the viability of cells was assessed. Table III presents the IC_{30} values of ACF for 24 h of treatment. In Fig. 2, the pattern of CRC cell responses to the cytotoxic effect of ACF + 5-FU is presented. In all ACF-pretreated cell lines, at the low concentration of 5-FU, an increased amount of cell death occurred. Furthermore, the IC_{50} value of 5-FU in the pretreatment protocol was significantly lower compared with the IC_{50} value of 5-FU when used as a single drug (Table IV). In fact, ACF pretreatment was able to sensitize CRC cells to the low concentration of 5-FU.

Irinotecan is one of the standard drugs routinely used in combination with 5-FU for the treatment of patients with CRC. CRC cells were pretreated with irinotecan using the same

Table II. Sensitivity of CRC cell lines to 5-FU when used as single agents or in combination (5-FU + ACF).

	IC_{50} value, μM		
CRC cell line	5-FU	5-FU + ACF	P-value
SW480	41.85±16.44	64.66±8.22	0.275
HCT116	7.36±4.14	11.59±1.80	0.465
LS174T	2.35±1.10	3.07±2.27	0.564

Results are IC_{50} values obtained when cells were treated for 72 h with different concentrations of 5-FU, and when cells were co-treated with the 30% maximal inhibitory concentration of ACF and different concentrations of 5-FU for 72 h. P-values were derived when 5-FU and 5-FU + ACF results for each cell line were statistically compared using a Mann-Whitney U test. Results are the mean ± standard deviation obtained from three independent assays, each performed in duplicate. CRC, colorectal cancer; 5-FU, 5-fluorouracil; ACF, acriflavine; IC_{50}, half-maximal inhibitory concentration.

Table III. IC_{30} values of ACF and irinotecan for CRC cell lines.

	IC_{30} value, μM	
CRC cell line	ACF	Irinotecan
SW480	4.85±1.03	56.97±4.52
HCT116	4.41±0.57	80.01±1.91
LS174T	3.10±0.24	33.12±3.06

Results are IC_{30} values obtained when cells were treated for 24 h with different concentrations of ACF or irinotecan. Results are the mean ± standard deviation obtained from three independent assays, each performed in duplicate. The calculated IC_{30} values were used for the pretreatment protocol. CRC, colorectal cancer; IC_{30}, 30% maximal inhibitory concentration; ACF, acriflavine.

protocol as for ACF, and the IC_{50} value of 5-FU was determined and compared with the IC_{50} value of 5-FU when used as a single agent or when pretreated with ACF. Irinotecan has an inhibitory effect on cell viability in a dose-dependent manner (data not shown). The IC_{30} values of irinotecan for 24 h of treatment are presented in Table III. Pretreatment with IC_{30} of irinotecan significantly increased the antitumor activity of 5-FU in CRC cells (Table IV). In comparison with irinotecan, ACF was identified to be a more potent agent for enhancing the antitumor activity of 5-FU (Table IV). CRC cells that were pretreated with ACF were significantly more sensitive to 5-FU compared with the cells pretreated with irinotecan (Table IV).

It is worthy of mention that the pretreatment protocol is independent of the co-treatment protocol and the two protocols were not compared.

Effects of ACF treatment on mRNA expression levels of HIF-1α and topoisomerase 2 in CRC cells. To assess the possible cytotoxic mechanism of ACF action, cells were treated with the IC_{30} of ACF for 24 h. qPCR was subsequently used to determine mRNA levels of HIF-1α and topoisomerase 2. The results identified that ACF did not significantly alter the mRNA levels of either HIF-1α or topoisomerase 2 (Fig. 3).

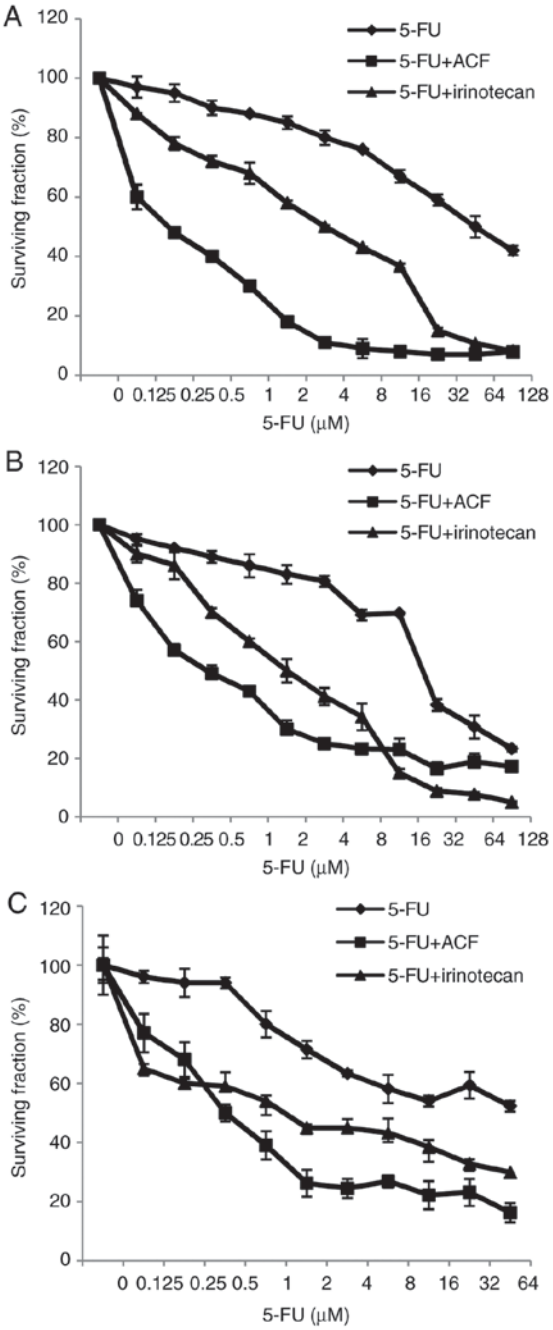

Figure 2. Comparison of the ability of 5-FU, 5-FU + ACF and 5-FU + irinotecan to inhibit the viability of (A) SW480, (B) HCT116 and (C) LS174T cell lines. Cells were treated with different concentrations of 5-FU as a single agent for 48 h, or pretreated with the 30% maximal inhibitory concentration of ACF or irinotecan for 24 h and then exposed to different concentration of 5-FU for 48 h. Cell viability was determined using an MTT assay. Results are the mean ± standard deviation of three experiments, each performed in triplicate. 5-FU, 5-fluorouracil; ACF, acriflavine.

Discussion

Conventional chemotherapy regimens have exhibited limited curative effects in CRC and a significant proportion of patients with advanced CRC exhibit resistance to chemotherapy (22,23).

Table IV. Sensitivity of colorectal cancer cell lines to 5-FU when used as a single agent or when pretreated with ACF (5-FU + ACF) and/or irinotecan (5-FU + irinotecan).

	IC_{50} value, μM					
CRC cell line	5-FU	5-FU + ACF	5-FU + irinotecan	P-value[a]	P-value[b]	P-value[c]
SW480	107.93±5.13	0.22±0.03	9.96±0.55	0.001	0.004	0.003
HCT116	35.44±1.04	0.28±0.06	2.20±0.38	0.007	0.009	0.006
LS174T	53.35±10.73	0.32±0.04	1.16±0.17	0.006	0.009	0.008

Results are IC_{50} values obtained when cells were in culture for 24 h and then treated for 48 h with different concentrations of 5-FU alone, or when cells were pretreated with the 30% maximal inhibitory concentration of ACF and/or irinotecan for 24 h and different concentrations of 5-FU for 48 h. P-values were derived when 5-FU results for each cell line were statistically compared using Kruskal-Wallis test and Dunn's post hoc test. Results are the mean ± standard deviation obtained from three independent assays, each performed in triplicate. [a]P-value for 5-FU vs. 5-FU + ACF. [b]P-value for 5-FU vs. 5-FU + irinotecan. [c]P-value for 5-FU+ACF vs. 5-FU+irinotecan. CRC, colorectal cancer; 5-FU, 5-fluorouracil; ACF, acriflavine; IC_{50}, half-maximal inhibitory concentration.

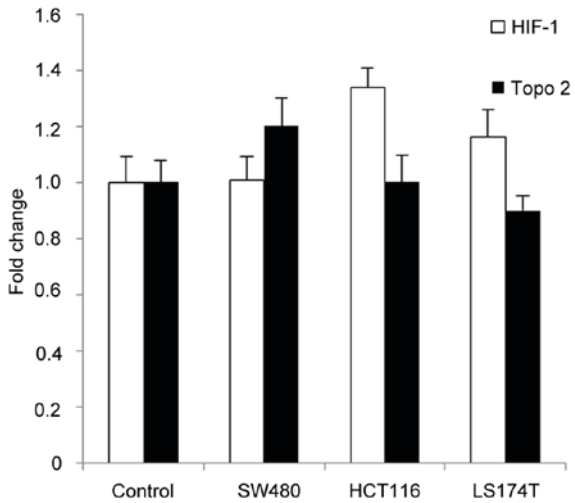

Figure 3. Effects of ACF on mRNA expression levels of HIF-1α and topoisomerase 2 in colorectal cancer cell lines. Cells were treated with the 30% maximal inhibitory concentration of ACF for 24 h and then total RNA was extracted and used for reverse transcription-quantitative polymerase chain reaction assay. Results are the mean ± standard deviation of three experiments, each performed in triplicate. ACF, acriflavine; Topo, topoisomerase.

5-FU is a first-line treatment in patients with advanced CRC. However, only a small proportion of patients respond to 5-FU when used as a single agent. Administration of other chemotherapy drugs in combination with 5-FU is reported to improve the response rate of patients (9,11).

In the present study, the effect of ACF on the anticancer activity of 5-FU in CRC cells was investigated. The results indicated that ACF inhibits the growth of three CRC cell lines (SW480, LS174T and HCT116) in a dose-dependent manner. Treatment of cells with different concentrations of ACF for 24 or 72 h indicated that SW480 cells have the most and LS174T cells have the least sensitivity to ACF. Anticancer effects of ACF on different cancer cell lines and in mouse models of cancer have been demonstrated previously (14-16). Hassan *et al* (19) demonstrated that ACF has a cytotoxic effect in monolayer and multicellular spheroid culture of CRC cells, and their results demonstrated that ACF has a good cellular penetration and cytotoxic activity against hypoxic CRC cells.

For advanced CRC, different combinations of drugs were used as chemotherapy regimens in which 5-FU was considered as the main drug. Because of the powerful selection that results in the eventual emergence of cellular resistance to chemotherapy drugs, the combinatory use of different agents is critical for the successful treatment of CRC. However, in the majority of previous studies, the anticancer activity of ACF against CRC was investigated as a single agent. In the present study, the effects of ACF on the sensitivity of CRC cells to 5-FU were investigated using two separate protocols. In the first protocol, CRC cells were co-treated with a low concentration (IC_{30}) of ACF and different concentrations of 5-FU for 72 h. The results revealed that ACF co-treatment was not able to improve the sensitivity of cells to 5-FU. In the second protocol, CRC cells were pretreated with the IC_{30} of ACF for 24 h, then the drug was omitted and various concentrations of 5-FU were added. Pretreatment with ACF significantly increased the antiproliferative effect of 5-FU in comparison with 5-FU alone. The IC_{50} value of SW480, the most resistant cell, for 5-FU was decreased from 107 to 0.22 μM following pretreatment with ACF. Additionally, ACF-pretreated CRC cells were significantly more sensitive to 5-FU than the cells pretreated with irinotecan, a standard chemotherapy drug that is routinely used along with 5-FU. These results imply that ACF is a more suitable agent compared with irinotecan for enhancing the efficacy of 5-FU-based chemotherapy. Weijer *et al* (24) demonstrated that pretreatment with ACF for 24 h improves the response of human perihilar cholangiocarcinomas cells to photodynamic therapy and decreases tumor cell survival. In addition, pretreatment of the HCT116 cell line with ACF has been demonstrated to potentiate radiation-induced cell death (25).

It has been identified that the loss of p53 function is associated with tumor resistance to 5-FU (26-28). The responses of cells and patients to 5-FU chemotherapy are dependent on p53 status, with cells and patients with a mutated form of p53 having a higher resistance to 5-FU chemotherapy (29,30). Disrupting both alleles of TP53 in a colon cancer cell line made the cells highly resistant to apoptosis induced by 5-FU (26).

Seth *et al* (31) demonstrated that restoration of wild-type p53 may overcome the drug resistance of human cancer associated with p53 dysfunction. In the present study, SW480 cells were used that express a mutated form of p53 (32) and two other cell lines, HCT116 and LS174T, with wild-type p53 (33). In the present study, SW480 cells exhibited an increased IC_{50} value and were more resistant to the cytotoxic effect of 5-FU compared with the other two cell lines. However, pretreatment of cells with ACF markedly sensitized all three cell lines to the anticancer effects of 5-FU, regardless of the p53 status of cells. Therefore, the combination of 5-FU and ACF, the naturally occurring product with low side effects, may be a promising strategy to increase 5-FU-mediated cytotoxicity even in patients with p53-mutated CRC.

It appears that the underlying molecular mechanism of the antitumor property of ACF varies depending on the type and origin of cancer. ACF may intercalate DNA and RNA, and inhibit nucleolar RNA synthesis and topoisomerase 2 activity (13). It has been identified that certain anticancer activities of ACF are associated with disruption of the cell-surface membrane that leads to protein kinase C inhibition (34). ACF may sensitize cells to chemotherapeutic agents through the suppression of the expression of xenobiotic-metabolizing genes (35). Furthermore, in previous studies, the effect of ACF on cell cycle (24), caspase activity (14,24,25) and expression of angiogenic genes (16) were investigated. In hepatocellular carcinoma cells, ACF induced apoptosis through the suppression of B-cell lymphoma 2 expression (14). ACF was also identified to bind HIF-1α and inhibit its transcriptional activity, which was associated with an inhibitory effect on tumor growth and vascularization in prostate cancer xenografts (36). In the present study, other effects of ACF that, to the best of our knowledge, have not been determined previously and were more associated with CRC were investigated.

As aforementioned, certain anticancer effects of ACF were associated with the inhibition of HIF-1α and topoisomerase 2 activity. Overexpression of HIF-1α has been identified to be involved in the pathogenesis of CRC (37,38). In previous studies of CRC, the effects of ACF on the expression of genes for HIF-1α and topoisomerase 2 by cells were not assessed. In the present study, the effects of ACF on the cellular expression of genes encoding HIF-1α and topoisomerase 2 were investigated using RT-qPCR. The results identified that pretreatment of cells with ACF was not able to significantly alter the expression of HIF-1α and topoisomerase 2. Therefore, it appears that the cytotoxic effect of ACF is not exerted through suppression of transcription of HIF-1α and topoisomerase 2 genes in CRC cells.

In the present study, the co-treatment protocol was not able to enhance the cytotoxicity of 5-FU, but pretreatment with ACF was able to significantly increase 5-FU cytotoxicity. The reasons for this are unclear. It appears that pretreatment with ACF predisposes CRC cells to the cytotoxic effect of the main drug, i.e. 5-FU, through the inhibition of HIF-1α and topoisomerase 2 activity as suggested previously (17,19). However, the exact molecular mechanism of ACF cytotoxicity against CRC cells remains to be elucidated (19). Additionally, it has been identified that 5-FU exerts its anticancer property primarily through the inhibition of thymidylate synthase (7). Therefore, investigating the effect of ACF on thymidylate synthase, which is a target of 5-FU, is a focus of future research. A limitation of the present study is that the cytotoxic effect of ACF and 5-FU on normal colon epithelial cell as a control was not determined.

Taken together, the results of the present study reveal for the first time that the pretreatment with a low concentration of a naturally occurring product, ACF, markedly increases the cytotoxic effects of 5-FU in CRC cells. This effect is independent of the p53 status of cells and is not exerted through the suppression of the expression of mRNAs for HIF-1α and topoisomerase 2 in CRC cells. The combination of ACF and 5-FU may be considered as a potential new chemotherapy regimen to overcome 5-FU resistance and improve the survival of patients with advanced CRC. However, for optimizing the ACF dose for the treatment of human CRC, other *in vivo* studies are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

PZ performed experiments and collected data. EG analyzed and interpreted data. AR analyzed data and performed experiments. FJM analyzed and interpreted data, prepared figures and wrote the introduction section. EE developed the concept and designed the study, and wrote the manuscript. All authors read and approved the final article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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