Synergistic antitumour effect of raltitrexed and 5-fluorouracil plus folinic acid combination in human cancer cells

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5-Fluorouracil, usually in combination with folinic acid, is widely used in the treatment of both colorectal and head and neck squamous cell cancer patients. Since 5-fluorouracil plus folinic acid and the antifolate thymidylate synthase inhibitor; raltitrexed have distinct mechanisms of action and toxicity profiles, we have evaluated the potential synergistic antitumor interaction between these two agents combined with a sequential schedule of administration in KB (wt-p53) and Cal27 (mut-p53) head and neck squamous cell carcinomas, and LoVo (wt-p53) and HT29 (mut-p53) colorectal cell lines. The combination between a 24-h exposure to raltitrexed followed by a 4-h exposure to 5-fluorouracil plus folinic acid was globally synergistic, as assessed by the median effect principle and combination index. A specific contribution of folinic acid to the cytotoxic effect of the raltitrexed/5-fluorouracil combination was clearly demonstrated by the evaluation of the potentiation factor. In all cell lines, a 1.5- up to 17-fold reduction in the IC_{50} of both raltitrexed and 5-fluorouracil plus folinic acid was observed in the combination setting compared with the concentrations of the each drug used alone. Moreover, we demonstrated that raltitrexed/5-fluorouracil plus folinic acid induced a distinct S-phase block of the cell cycle, as well as a potentiation of the apoptotic cell death, compared with 5-fluorouracil plus folinic acid or raltitrexed/5-fluorouracil combination. This preclinical work represents, at least to our knowledge, the first demonstration of a synergistic interaction between raltitrexed and 5-fluorouracil modulated by folinic acid, and could represent a rationale for further clinical investigation of raltitrexed/5-fluorouracil plus folinic acid combination. *Anti-Cancer Drugs* 18:781–791 © 2007 Lippincott Williams & Wilkins.

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Introduction

5-Fluorouracil (5FU) is widely used in the treatment of a variety of human malignancies, including colorectal cancers (CRC) and head and neck squamous cell carcinomas (HNSCC) [1,2]. The mechanism of cytotoxicity of 5FU has been ascribed to the misincorporation into RNA and DNA of its active metabolites 5-fluorouridine triphosphate (5FUTP) and 5-fluorodeoxyuridine triphosphate (5FdUTP), respectively, and to the inhibition of thymidylate synthase (TS) by 5-fluorodeoxyuridine monophosphate (5FdUMP). TS has a key role in DNA synthesis, catalyzing the conversion of deoxyuridine monophospate (dUMP) into thymidylate (dTMP), with 5,10-methylene tetrahydrofolate (CH_2THF) playing as a methyl donor, this reaction being the sole intracellular source of dTMP. FdUMP inhibits dTMP synthesis by forming a stable ternary complex with TS and CH_2THF, thereby blocking the binding of the normal substrate dUMP [2,3]. Folinic acid (FA), a precursor for CH_2THF, increases the formation of the stabilized ternary complex when associated with 5FU, resulting in enhanced cytotoxicity in preclinical models [4], as well as in a significant improvement in response rate and overall survival in advanced CRC [5].

A novel therapeutic approach has been recently proposed with the development of direct and selective antifolate TS inhibitors such as the quinazoline raltitrexed (RTX). RTX, the first antifolate drug to undergo extensive clinical evaluation, is transported into cells via the reduced folate carrier and is then extensively polyglutamated, acquiring up to 100-fold drug potency in comparison with the nonpolyglutamated compound [6]. Clinical trials demonstrated that RTX is active in a number of solid tumours, including CRC and HNSCC [7,8]. In detail, RTX showed similar response rates to bolus 5FU plus FA (5FU–FA) in patients with advanced CRC, but induced less neutropenia and mucositis [9,10]. Therefore, RTX has been licensed in many countries as an active agent for the treatment of metastatic CRC. The

ease modality of delivery and its favourable toxicity profile, however, make RTX an ideal agent for combinatorial use, and several clinical studies have been carried out in which RTX has been combined with other agents such as irinotecan and oxaliplatin [11–13].

Preclinical and clinical studies have demonstrated a strong association between the increased TS expression and the development of both 5FU and RTX resistance [2,14–16]. Furthermore, exposure of cancer cells to 5FU or other TS inhibitors acutely upregulates TS synthesis and the latter effect appears to be due to the inhibition of a negative-feedback mechanism, in which TS binds its own mRNA and inhibits translation [2,3,17,18]. In addition, TS, which has recently been reported to have an oncogene-like activity [19], can bind and decrease the expression of genes involved in the regulation of proliferative and survival pathways such as c-*myc* and p53 [20,21]. On the other hand, expression of wild-type p53 has been shown to be required for 5FU-induced apoptosis *in vitro* [22,23], whereas several reports demonstrated that RTX-induced apoptosis appears either p53-indipendent [23–25] or at least less dependent on p53 expression as compared with 5FU [26].

In-vitro data as well as clinical studies have shown synergism and positive pharmacokinetic interaction between RTX and 5FU in CRC, when a sequential schedule of RTX followed by 5FU was used [27,28]. Interestingly, preclinical antitumour synergism as well as pharmacokinetic interaction was obtained when a short exposure to 5FU was used. In contrast, it has been shown that in-vitro cytotoxicity of RTX is completely antagonized by the coincubation with FA. The rescue effect, however, is abolished when FA exposure is delayed by 24 h [29].

In this study, we have evaluated the in-vitro antitumour effects exerted by a 24-h exposure to RTX followed by a 4-h exposure to 5FU–FA in human HNSCC and CRC cell lines. We have used the combination index (CI) analysis method [30–33] for the evaluation of the drug effect interaction on mutant (mut) and wild-type (wt) p53 cells for each tumour type. Additional investigations into biochemical modulation of TS expression as well as cell cycle analysis and apoptosis detection have also been performed. This preclinical work paralleled the clinical investigation we have carried out on RTX plus 5FU–FA combination in both HNSCC and CRC patients [34–36].

Materials and methods

Drugs

RTX was supplied by AstraZeneca (Macclesfield, UK), 5FU was supplied by Teva Pharmaceutical Industries (Netanya, Israel) and FA was obtained from Wyeth Pharma (Münster, Germany).

Cell culture and cell proliferation assay

KB, HT29 and LoVo cell lines were from the American Type Culture Collection (Rockville, Maryland, USA), CAL27 cell line was kindly provided by Dr JL Fishel (Centre A Lacassagne, Nice, France). KB, HT29 and CAL27 cell lines were grown in Dulbecco's modified Eagle's medium, whereas LoVo cells were grown in Roswell Park Memorial Institute (RPMI 1640). Both media were supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, 500 µg/ml streptomycin and 4 mmol/l glutamine in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. All media, serum, antibiotics and glutamine were from Cambrex Bio Science (Verviers, Belgium).

Cell survival/proliferation was measured in 96-well plates by a spectrophotometric dye incorporation assay (sulforhodamine B) as described previously [37]. Treatment schedule was performed throughout all the experiments as follows: 24 h after seeding (1×10^3 cells/well) cells were exposed to RTX for 24 h and/or 48 h after seeding cells were exposed to 5FU or 5FU–FA for 4 h; then a medium change was performed. Cell growth assessment was performed after 96 h, after the beginning of exposure to RTX (72 h after the beginning of treatment with 5FU or 5FU–FA). As FA did not exhibit any effect on cell proliferation when given alone and no increased effect was obtained by using doses higher than 10 µmol/l in combination with 5FU, all further combination experiments were performed at this FA dose level and 5FU–FA was considered as a single drug. Experimental conditions were tested in quadruplicates3 and experiments were performed at least three times for each cell line. For combination studies, cells were exposed to RTX for 24 h, then after a medium change cells were exposed for an additional 4 h to 5FU or 5FU–FA and then another medium change was performed. Cell growth assessment was performed after an additional 68 h (96 h after beginning exposure to RTX or 72 h after beginning treatment with 5FU or 5FU–FA).

Drug combination study

The CI was calculated by the Chou–Talalay equation, which takes into account both the potency (D_m or IC_{50}) and the shape of the dose–effect curve [30,31], taking advantage of the CalcuSyn software (Biosoft, Cambridge, UK).

The general equation for the classic isobologram (CI = 1) is given by $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + [(D_x)_1\bullet(D_x)_2]/[(D)_1 + (D)_2]$, where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) for D_1 (drug 1) and D_2 (drug 2) alone that gives x% inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the doses of drug 1 and drug 2 in combination, which also inhibited x% (i.e. isoeffective). $CI < 1$, $CI = 1$ and $CI > 1$ indicate

synergism, additive and antagonism effect, respectively. We, however, decided to use a cut-off for the CI of 0.8 as described previously [29], and identified a synergistic effect when $CI \leq 0.8$, an antagonistic effect when $CI \geq 1.2$, and an additive effect when CI was between 0.8 and 1.2.

The linear correlation coefficient (r) of the median-effect plot is considered the first line of statistics to measure the conformity of the data with the mass-action law principle when the experimental measurement is assumed to be accurate. A r value of 1 indicates perfect conformity. A poor r value may be the result of biological variability or experimental deviations. For tissue culture systems a minimum r value of 0.90 should be obtained to consider the experiment valid. Throughout all the experiments we obtained a mean $r = 0.95 \pm 0.01$, as reported also in Table 1.

The dose reduction index represents the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone.

Drug combination studies *in vitro* were based on concentration–effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentrations. First, the drugs at their equipotent ratio (e.g. at the ratio of their IC_{50}s, 50 : 50 cytotoxic ratio) were chosen and a dilution of each drug (RTX and 5FU or 5FU–FA) at four-fold (or eight-fold) of their IC_{50}s were made and serially diluted to obtain a good dosage range (Calcusyn, software manual). To explore the relative contribution of each agent to the synergism, two other mixtures of the two drugs (RTX and 5FU or 5FU–FA) were tested in each cell line: higher relative doses of RTX, resulting in about 75% of cell kill, with lower doses of 5FU or 5FU–FA, resulting in 25% of cell kill (75 : 25 cytotoxic ratio); and lower relative doses of RTX, resulting in about 25% of cell kill, with higher doses of 5FU or 5FU–FA, resulting in 75% of cell kill (25 : 75 cytotoxic ratio).

Furthermore, we analysed the specific contribution of FA on the cytotoxic effect of the RTX/5FU combination by calculating the potentiation factor (PF), defined as the ratio of the IC_{50} of RTX + 5FU to the IC_{50} of RTX + 5FU–FA combinations as described before; a higher PF indicates a greater cytotoxicity [38].

Protein extraction and Western blotting

Cells, grown and treated as described before, were lysed as reported previously [31]. An equal amount of protein, monitored by the Lowry assay using bovine serum albumin as standard, was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose paper, immunoblotted with specific antibody and probed with the appropriate horseradish peroxidase-linked IgG. Immunoreactive bands were detected by enhanced chemiluminescence system. Anti-TS antibody was from Rockland (Pennsylvania, USA) and anti-p53 antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). The nitrocellulose blot used for the analysis of TS expression was stripped at 50°C in stripping solution (100 mmol/l β-mercaptoetanol, 2% sodium dodecyl sulphate, 62.5 mmol/l Tris–HCl, pH 6.7) and then incubated overnight with antiglyceraldehyde-3-phosphate dehydrogenase antibody (Cell Signalling Technology, Massachusetts, Boston, USA) as loading control.

Analysis of cell cycle kinetic and apoptosis

For the evaluation of cell cycle kinetics and apoptotic cell death, cells were exposed to RTX for 24 h, then, after a medium change, cells were exposed for additional 4 h to 5FU or 5FU–FA and then another medium change was

Table 1 **CIs values[a] according to the different cytotoxic ratio of RTX/5FU or RTX/5FU–FA combinations**

Cell lines	Tissue of origin (p53 status)	RTX/5FU			RTX/5FU–FA		
		50 : 50	25 : 75	75 : 25	50 : 50	25 : 75	75 : 25
		CI_{50} (± SD) r (± SD)	CI_{50} (± SD) r (± SD)	CI_{50} (± SD) r (± SD)	CI_{50} (± SD) r (± SD)	CI_{50} (± SD) r (± SD)	CI_{50} (± SD) r (± SD)
KB	Head and neck (p53 wild-type)	0.86 (0.45) 0.94 (0.004)	0.81 (0.24) 0.96 (0.01)	0.96 (0.06) 0.95 (0.02)	0.85 (0.19) 0.96 (0.02)	0.69 (0.08) 0.98 (0.007)	1.1 (0.3) 0.95 (0.02)
Cal27	Head and neck (p53 mutant)	0.86 (0.22) 0.97 (0.02)	0.94 (0.07) 0.95 (0.007)	0.8 (0.1) 0.96 (0.009)	0.7 (0.02) 0.97 (0.01)	0.67 (0.18) 0.95 (0.01)	0.79 (0.09) 0.96 (0.03)
HT29	Colon (p53 mutant)	0.7 (0.01) 0.95 (0.01)	0.54 (0.16) 0.98 (0.02)	0.82 (0.05) 0.94 (0.008)	0.52 (0.03) 0.98 (0.002)	0.49 (0.22) 0.94 (0.006)	0.78 (0.08) 0.94 (0.01)
LoVo	Colon (p53 wild-type)	0.57 (0.18) 0.95 (0.03)	0.63 (0.09) 0.97 (0.009)	0.74 (0.1) 0.94 (0.01)	0.43 (0.09) 0.94 (0.006)	0.42 (0.12) 0.96 (0.008)	0.71 (0.21) 0.96 (0.004)

[a]CI values (mean ± SD from at least three separate experiments performed in quadruplicates) computed at 50% of cell kill (CI_{50}) by CalcuSyn software. 50 : 50 cytotoxic ratio, evaluation of CIs at equipotent doses of the two agents [RTX (IC_{50})/5FU or 5FU–FA (IC_{50})]; 25 : 75 cytotoxic ratio, evaluation of CIs at higher relative doses of 5FU or 5FU–FA [RTX (IC_{25})/5FU or 5FU–FA (IC_{75})].

CIs less than 0.8 indicated synergism, greater than 1.2 antagonism, and between 0.8 and 1.2 additivity.

The linear correlation coefficient (r) for the fitting between CIs and fractional effects was always between 0.94 and 0.98, indicating that the data were statistically accurate.

5FU, 5-fluorouracil; FA, folinic acid; RTX, raltitrexed.

performed. Cytofluorimetric analysis of cell cycle and apoptosis were performed after an additional 48 or 72 h, respectively, upon propidium iodide labelling, as reported previously [31]. These experiments were designed to study cell cycle kinetics and apoptotic cell death at the same time point of the antiproliferative study (72 h) or 24 h before (48 h).

Statistical analysis

The Friedman nonparametric rank test was used to compare the different cytotoxic ratios of RTX + 5FU–FA combinations (50 : 50, 75 : 25 and 25 : 75) based on of the PFs. The *P*-values were computed using SPSS software (SPSS), Chicago, Illinois, USA). A *P*-value less than 0.05 was considered to be statistically significant.

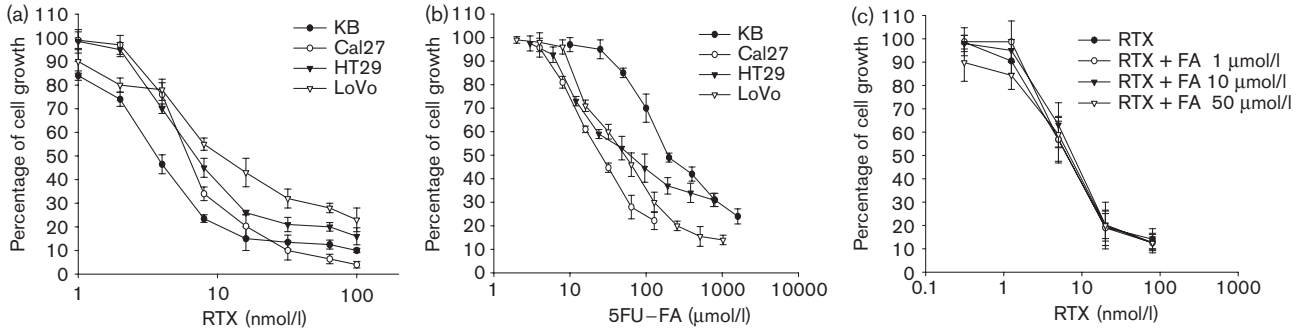
The Student's *t*-test was carried out on apoptotic cell death values data using Sigmastat software (www.systat.com) to assess statistically significant differences between RTX/5FU–FA combination and RTX/5FU or 5FU–FA. *P*-values are indicated and were considered to be statistically significant if less than 0.05.

Results

Raltitrexed and 5-fluorouracil induced cytotoxic effects in head and neck cancer and colorectal cancer cells as single agents or in combination with folinic acid

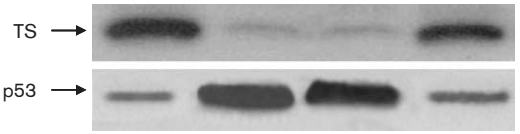
We have examined the cytotoxic effect of RTX, 5FU and 5FU–FA on four human cancer cell lines with different p53 status and TS expression and different tissue of origin: KB (wt-p53) and Cal27 (mut-p53) HNSCC cells, and LoVo (wt-p53) and HT29 (mut-p53) CRC cells. An inverse association was found between TS and p53 expression, with mut-p53 Cal27 and HT29 cells expressing a higher level of p53 and lower level of TS as compared with wt-p53 KB and LoVo cells (Fig. 1d). Multiple concentrations of FA in combination with 5FU were tested; however, as no increased cytotoxic effect was obtained using doses higher than 10 μmol/l (data not shown),

Fig. 1



(d) IC_{50}values (±SD)

	KB	Cal27	HT29	LoVo
RTX (nmol/l)	4±0.2	6±0.3	7±0.2	11±0.4
5FU (μm/l)	300±10	40±4	100±90	70±4
5FU (μmol/l) (modulated by FA 10 μmol/l)	195±4	26±0.5	65±3.2	55±2.2



Cytotoxic effect of RTX, 5FU, 5FU–FA, or RTX plus FA, and TS and p53 expression, in KB, Cal27, HT29 and LoVo cells. (a and b) Dose–response curves of RTX (24 h of exposure) and 5FU–FA (4 h of exposure) cytotoxic effects in all four cell lines. (c) Dose–response curves of RTX (24 h of exposure) alone or plus different doses of FA (4 h of exposure) cytotoxic effects in HT29 cells. Cell growth assessment, expressed as percentage of control, was performed 96 h after beginning treatment with RTX or 72 h after beginning treatment with FA, 5FU or 5FU–FA, as described in Materials and methods. Values were the mean of at least five different experiments performed in quadruplicates. (d) IC_{50} values (± SD) for RTX, 5FU and 5FU–FA, evaluated as described before; and Western blot analysis of TS and p53 expression performed on whole-cell protein lysates (70 μg) separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and immunoblotted with specific anti-TS or anti-p53 antibodies. 5FU, 5-fluorouracil; FA, folinic acid; RTX, raltitrexed; TS, thymidylate synthase.

all further combination experiments were performed at this FA dose level.

Dose–response curves demonstrated that all cell lines were growth-inhibited by both RTX (24-h exposure) and 5FU-FA (4-h exposure) (Fig. 1a and b) with a different pattern. LoVo cells were the most resistant to RTX (IC_{50} = 11.2 nmol/l) and KB the most resistant to 5FU modulated by FA (IC_{50} = 195 μmol/l) (Fig. 1a, b and d). As indicated in Fig. 1d, addition of FA 10 μmol/l induced a decrease of the IC_{50} values of 5FU ranging from 21 to 35%.

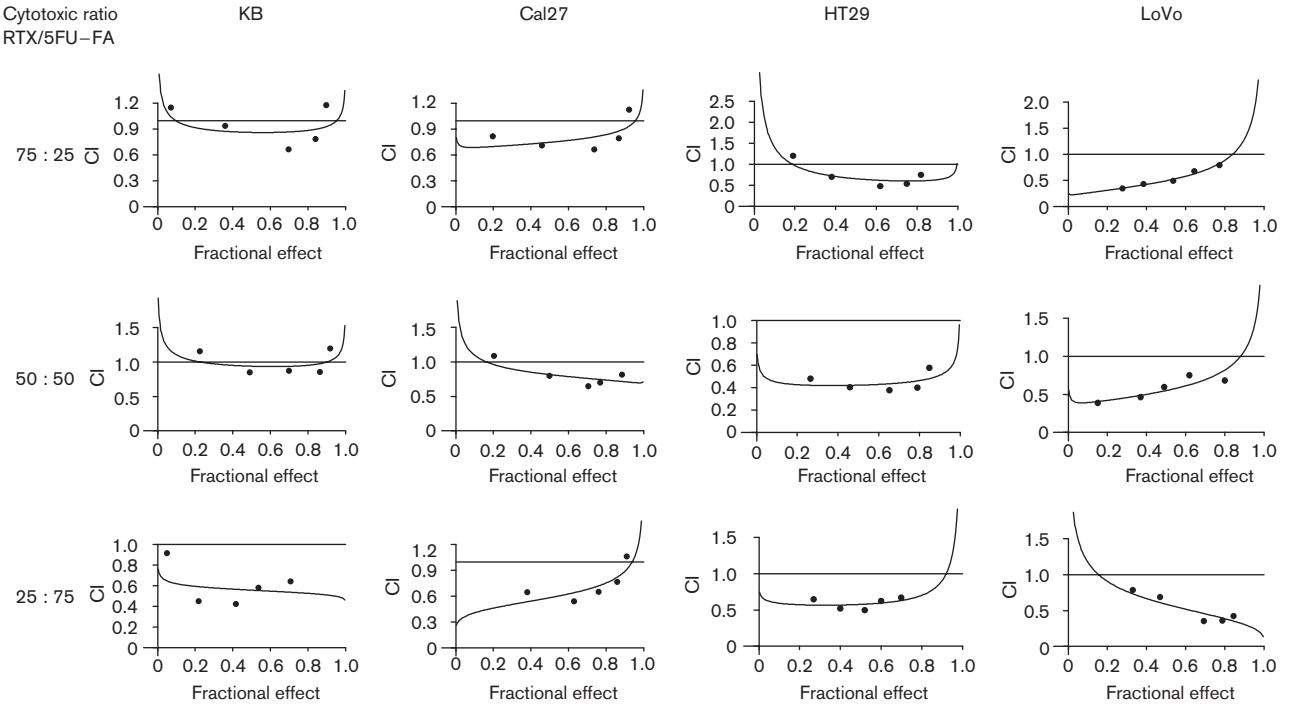
We have also shown that multiple concentrations of FA added 24 h after RTX do not affect RTX-induced cytotoxicity in HT29 cells (Fig. 1c) as well as in all other cell lines (data not shown). The latter observation confirmed that FA treatment should be delayed by 24 h after RTX exposure to abolish the rescue effect described before [29].

Combination treatment of raltitrexed plus 5-fluorouracil-folinic acid resulted in synergistic cytotoxic effect

As reported previously, 5FU should follow RTX treatment by 24 h to obtain a better antitumour effect in the combination setting [27]. On the basis of this premise, we have studied, on the four HNSCC and CRC cell lines, the combination treatment schedule of 24-h exposure to RTX, followed by 4-h exposure to 5FU or 5FU–FA and we calculated the CI values, as described in Materials and methods [30,31]. In details, to explore the relative contribution of each agent to the synergism, serial dilutions from three different mixtures of the two drugs (RTX plus 5FU or 5FU–FA) were tested using equipotent doses of the two agents (50 : 50 cytotoxic ratio), higher relative doses of RTX (75 : 25 cytotoxic ratio) and higher relative doses of 5FU or 5FU–FA (25 : 75 cytotoxic ratio). Typical examples of CI/fractional effect curves showing the CIs versus the fraction of cells affected/killed by RTX and 5FU–FA in combination are illustrated in Fig. 2 for the different cell lines.

The CI values, computed at the 50 : 50, 25 : 75 and 75 : 25 cytotoxic ratios and calculated at concentrations resulting in 50% of cell kill (CI_{50}) for both RTX/5FU and RTX/5FU–FA, are reported in Table 1. The analysis of CIs revealed that RTX/5FU–FA combinations were globally synergistic (CIs < 0.8), independently of the cytotoxic ratio used, in three cell lines but not in KB cells. In the latter cell line, a clear synergistic effect was observed only when RTX was combined with 5FU–FA at the 25 : 75 cytotoxic ratio, whereas only an additive effect was observed under different conditions (Fig. 2, Table 1).

Fig. 2



Typical examples of CI/fractional effects curves for KB, Cal27, HT29 and LoVo cells. Different cytotoxic ratios of RTX and 5FU–FA have been evaluated as described in Materials and methods. CIs less than 0.8 indicated synergism, greater than 1.2 antagonism, and between 0.8 and 1.2 additivity. CI, combination index; 5FU, 5-fluorouracil; FA, folinic acid; RTX, raltitrexed; TS, thymidylate synthase.

Table 2 Dose reduction index (DRI) values[a] according to the different cytotoxic ratio of RTX/5FU or RTX/5FU–FA combinations and potentiation factor (PF) values[b] by FA

Cell lines	50 : 50 cytotoxic ratio			25 : 75 cytotoxic ratio			75 : 25 cytotoxic ratio		
	DRI_{50} (±SD)		PF (±SD)[b]	DRI_{50} (±SD)		PF (±SD)	DRI_{50} (±SD)		PF (±SD)
	RTX/5FU	RTX/5FU-FA		RTX/5FU	RTX/5FU-FA		RTX/5FU	RTX/5FU-FA	
KB	2.36 (1.34)	2.4 (1.51)	1.08 (0.02)	7.7 (1.6)	10.5 (0.9)	2.2 (1.09)	1.2 (0.15)	1.2 (0.35)	1.03 (0.3)
	4.33 (3.88)	2.1 (1.07)		1.53 (0.28)	1.82 (0.44)		5.1 (1.4)	2.7 (0.45)	
Cal27	1.87 (0.43)	2.45 (0.57)	1.99 (1.16)	4.35 (0.07)	7.85 (1.5)	3.3 (1.2)	1.4 (0.18)	1.65 (0.3)	1.3 (0.4)
	3.7 (1.59)	3.55 (0.9)		1.5 (0.01)	1.95 (0.49)		4.3 (1.7)	4.8 (0.2)	
HT29	2.3 (0.56)	3.8 (0.14)	3.13 (1.74)	9.4 (0.8)	17 (0.88)	3.8 (3.1)	1.8 (0.2)	2.5 (0.8)	1.7 (0.2)
	4.35 (2.05)	3.9 (0.42)		3.16 (1.5)	3.04 (1.44)		2.9 (1.2)	2.3 (0.9)	
LoVo	9.0 (1.18)	11.0 (0.09)	2.59 (0.15)	10.7 (3.88)	13.9 (2.68)	5.76 (2.6)	1.95 (0.4)	4.7 (2.8)	1.5 (0.4)
	3.6 (0.98)	3.75 (1.16)		2.02 (0.89)	2.13 (1.41)		4.5 (0.28)	3.98 (1.43)	

[a]DRI values (mean ± SD from at least three separate experiments performed in quadruplicates) represent the order of magnitude (fold) of dose reduction obtained for IC_{50} (DRI_{50}) in a combination setting compared with each drug alone. 50 : 50 cytotoxic ratio, equipotent doses of the two agents [RTX (IC_{50})/5FU or 5FU–FA (IC_{50})]; 25 : 75 cytotoxic, evaluation of CIs at higher relative doses of 5FU or 5FU–FA [RTX (IC_{25})/5FU or 5FU–FA (IC_{75})]; 75 : 25 cytotoxic, evaluation of CIs at higher relative doses of RTX [RTX (IC_{75})/5FU or 5FU–FA (IC_{25})].

[b]PF values (mean ± SD from at least three separate experiments performed in quadruplicates) defined the specific contribution of FA evaluated as the ratio of the IC_{50} of RTX 5FU to the IC_{50} of RTX 5FU–FA. The Friedman nonparamentric rank test was used to analyze the impact of the different cytotoxic ratios on the whole cell line panel, $P = 0.018$.

FU, 5-fluorouracil; FA, folinic acid; RTX, raltitrexed.

Notably, in all cell lines, a 1.5- up to 17-fold reduction in the IC_{50} of both RTX and 5FU–FA (DRI_{50}) was observed in the combination setting compared with the concentrations of the drugs used alone (Table 2). Similar patterns were obtained for the RTX/5FU combination, with a slightly lower synergism (higher CI values) compared with RTX/5FU–FA in HT29 and LoVo cell lines, and an additive effect in KB and Cal27 cells (Table 1).

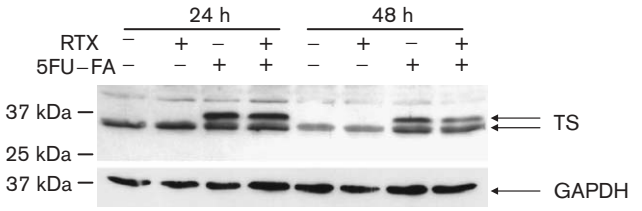
Moreover, values of PF reported in Table 2 demonstrated that FA had an important contribution to the cytotoxic effect of RTX/5FU combination in all cells. Interestingly, the optimal results (lowest CI values with the best PF) were obtained with a 25 : 75 cytotoxic ratio. Statistical analyses indicated that the PFs were significantly different depending upon the cytotoxic ratio ($P = 0.018$). All the following experiments were performed using a 25 : 75 cytotoxic ratio between RTX and 5FU or 5FU–FA.

Raltitrexed and 5-fluorouracil–folinic acid in combination induced biochemical modulation of thymidylate synthase

The increase in TS levels in cell lines after exposure to 5FU or antifolate TS inhibitors, such as RTX, seems to be a common finding. In addition, as described previously, 5FU induced inhibition of TS by the formation of a ternary complex between FdUMP, the enzyme and CH_2THF [3].

To evaluate if in our cell models RTX and 5FU–FA, as single agents or in combination, were able to modulate the expression and function of TS, we performed Western blotting analysis of TS protein expression on HT29 cells, expressing basal low level of TS.

Fig. 3



Western blotting analysis of TS expression in HT29 cells. Whole-cell protein lysates (70 μg) from cells untreated or treated with RTX (0.3 nmol/l), 5FU–FA (3 μmol/l) or RTX/5FU–FA combination, as described in Materials and methods, were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, immunoblotted with specific anti-TS antibody and reprobed with anti-GAPDH antibody to account for loading differences. CI, combination index; 5FU, 5-fluorouracil; FA, folinic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RTX, raltitrexed; TS, thymidylate synthase.

As shown in Fig. 3, a clear ternary complex was induced by 5FU–FA within 24 h and was maintained up to 48 h, as shown by the induction of the upper 38-kDa band [3]. The formation of the ternary complex is also achieved upon RTX/5FU–FA combination treatment indicating that RTX does not affect the biochemical inhibition of TS induced by 5FU.

The synergistic cytotoxic effect of raltitrexed and 5-fluorouracil–folinic acid in combination was paralleled by cell cycle perturbation

With the aim of investigating the mechanism of RTX/5FU–FA synergistic interaction, the cell cycle kinetic analysis was performed in all cell lines treated with each drug alone or in combination at the same time point of the antiproliferative study (72 h) or 24 h before (48 h).

Typical examples of histograms of DNA distribution for KB cells after 72 h of treatment are reported in Fig. 4. Low doses of RTX induced only a faint increase of S-phase percentage (S-phase cells = 26%) compared with the control KB cells (S-phase cells = 17%), whereas 5FU at IC_{50} concentration arrested cells in early mid-S phase (S-phase cells = 41%) and FA addition (5FU–FA), which alone do not induce any cell cycle perturbation (data not shown; S-phase cells = 18%), increased the percentage of cells arrested in mid-S phase (S-phase cells = 68%). Interestingly, the addition of low doses of RTX (RTX/5FU–FA) induced a slight increase of this effect (S-phase cells = 72%).

Notably, in RTX/5FU-treated cells we demonstrated a different mid-S-phase arrest (S-phase cells = 67%) compared with the shape of the cell cycle S-phase arrest observed in KB cells treated by the RTX/5FU–FA combination.

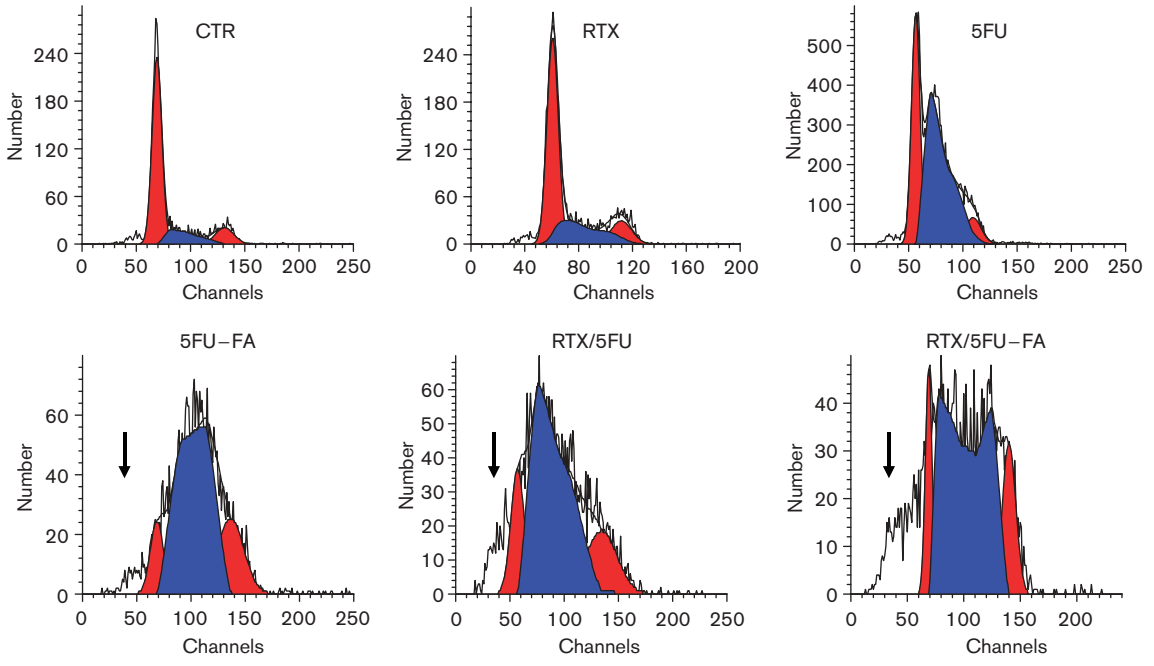
As shown in Table 3, in all cell lines a slightly more prominent S-phase block was demonstrated with the RTX/5FU–FA combination compared with 5FU–FA-treated cells after 48 h. The S-phase block was still evident after 72 h.

A similar pattern of S-phase cell cycle block was demonstrated in all cell lines also in RTX/5FU-treated cells, although it was globally less than that observed in RTX/5FU–FA combinations (data not shown).

Raltitrexed and 5-fluorouracil–folinic acid in combination induced apoptosis

To investigate further the mechanism of the synergism observed upon RTX/5FU–FA combination treatment, we performed cytofluorimetric analysis, after propidium iodide labelling, of apoptotic cell death in all four cell lines after 72 h (Fig. 5). Low doses of RTX did not induce a marked apoptotic effect compared with untreated cells, whereas increased apoptosis was observed in 5FU, 5FU–FA or RTX/5FU-treated cells. RTX/5FU–FA combination, however, induced an evident potentiation of the apoptotic cell death in all cell lines (Fig. 5). In detail, the apoptosis induced by RTX/5FU–FA combination was significantly higher than that induced by RTX/5FU or 5FU–FA in KB ($P \leq 0.001$ and $P \leq 0.001$), Cal27 ($P \leq 0.001$ and $P = 0.001$), HT29 ($P \leq 0.018$ and $P = 0.001$) and LoVo ($P \leq 0.04$ and $P = 0.008$) cells. This pattern is also shown in Fig. 4 (arrows), where a more prominent hypodiploid population (sub-G_0–G_1) appeared in RTX/5FU–FA compared with 5FU–FA or RTX/5FU-treated KB cells.

Fig. 4



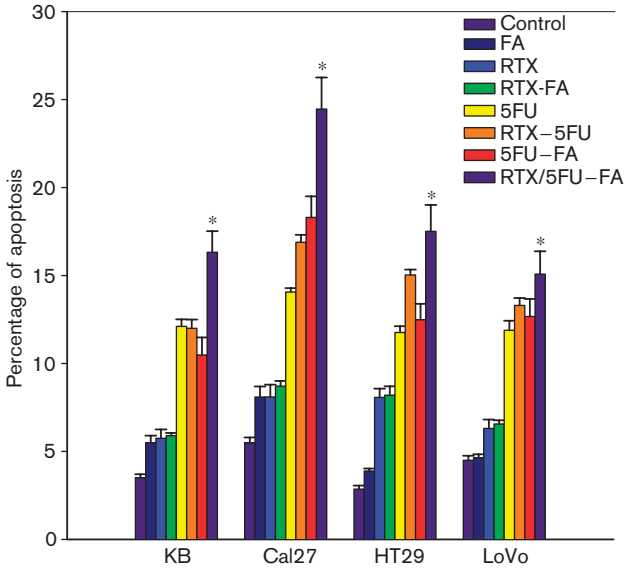
Cytofluorimetric analysis of cell cycle kinetics in KB cells. DNA distribution histogram using propidium iodide labelling (*x*-axis) and total number of cells in each channel (*y*-axis). KB cells untreated or treated as indicated (RTX 1.5 nmol/l, 5FU 100 μmol/l, FA 10 μmol/l) were collected 96 h after beginning treatment with RTX or 72 h after beginning treatment with 5FU or 5FU–FA, labelled with propidium iodide and analyzed by fluorescence-activated cell sorting as described in Materials and methods. Arrows indicate hypodiploid population (sub-G_0–G_1). Control cells (CTR), 5FU, 5-Fluorouracil; FA, folinic acid; RTX, raltitrexed.

Table 3 Effects of RTX and 5FU–FA alone or in combination on cell cycle distribution in KB, Cal27, HT29 and LoVo cells after 48 and 72 h of treatment at the indicated concentrations[a]

Cell lines	48 h			72 h		
	% G_0/G_1	%S	% G_2–M	% G_0/G_1	% S	% G_2–M
KB						
Control	55	25	20	71	17	12
RTX (1 nmol/l)	47	34	19	61	26	13
5FU–FA (100 μm/l)	43	49	8	11	68	21
RTX/5FU–FA	30	57	13	12	72	16
Cal27						
Control	66	18	16	67	19	14
RTX (3 nmol/l)	64	22	14	39	45	16
5FU–FA (12 μm/l)	31	42	27	12	75	13
RTX/5FU–FA	29	48	23	9	78	13
HT29						
Control	55	34	11	76	14	10
RTX (3 nmol/l)	44	41	15	71	13	16
5FU–FA (12 μm/l)	29	50	21	12	69	19
RTX/5FU–FA	20	63	17	20	75	5
LoVo						
Control	40	44	16	57	30	13
RTX(2 nmol/l)	35	33	32	20	40	40
5FU-FA (20 μm/l)	45	46	9	30	60	10
RTX/5FU–FA	22	72	6	15	73	12

[a]The values were means from at least three independent experiments and SDs were always less than 10%.
5FU, 5-fluorouracil; FA, folinic acid; RTX, raltitrexed.

Fig. 5



Cytofluorimetric analysis of apoptotic cell death in KB, Cal27, HT29 and LoVo cells. Treatment was performed as described in Materials and methods with different concentrations of drugs in each cell line: KB cells, RTX 1.5 nmol/l, 5FU 100 μmol/l; Cal27 cells, RTX 3 nmol/l, 5FU 12 μmol/l; HT29 cells, RTX 3 nmol/l, 5FU 12 μmol/l; LoVo cells, RTX 2 nmol/l, 5FU 20 μmol/l; FA was always 10 μmol/l. Cells were collected, labelled and analysed as in Fig. 4. The data represent the means of percentage of apoptotic cells calculated in the hypodiploid region of the DNA content, registered as FL3 signals in a log scale for at least three different experiments. Error bars: ± SD. The significance of differences between RTX/5FU–FA and either 5FU–FA or RTX/5FU was calculated using the paired Student's *t*-test with significance being accepted for $*P<0.05$. 5FU, Control cells (CTR), 5-fluorouracil; FA, folinic acid; RTX, raltitrexed.

Discussion

Previously reported in-vitro studies have shown a synergistic activity when RTX is followed 24 h later by 5FU as a short 4-h exposure [27], whereas a phase I study has also demonstrated a positive pharmacokinetic interaction between RTX and 5FU [28]. A positive effect of FA on 5FU antitumour activity has been demonstrated by several preclinical and clinical studies [5,6]. In contrast, FA, as a reduced folate cofactor, competes with RTX for the transport and polyglutamation in both normal and tumour tissues, and thus it has a potential rescuing effect. In fact, in-vitro cell studies have shown that the cytotoxic effects of RTX can be reversed by the simultaneous administration of FA, due to inhibition of drug uptake and intracellular polyglutamation [29,39]. An observation by Farrugia *et al.* [16], however, showed, *in vitro*, that the rescue effect on RTX-induced cytotoxicity was abolished if the addition of FA was performed 24 h later [29]. We confirmed these data demonstrating that multiple increasing doses of FA did not affect RTX-induced cytotoxicity or apoptosis *in vitro* when added 24 h later.

Subsequently, on these bases, in four HNSCC and CRC cell lines with different p53 status or TS expression, we have demonstrated a clear synergistic cytotoxic effect when RTX treatment for 24 h was followed by exposure to 5FU–FA for 4 h, as assessed by median effect principle and CI. We have also demonstrated a reduction of the IC_{50} of both RTX and 5FU–FA in combination (ranging from 1.5- to 17-fold), compared with the concentrations of each drug added alone, in all cell lines.

Moreover, a specific contribution of FA to the cytotoxic effect of RTX/5FU combination was clearly demonstrated by the evaluation of the PF. Although several previous papers described in-vitro combinations and mechanistic interactions between RTX and 5FU [27,40–42], confirmed in our cell models, the present in-vitro study is the only one, at least at our knowledge, to challenge with different approaches the role of FA in the potentiation of RTX/FU synergistic antitumour effect.

Previous observations reported that the presence of high folate levels in the medium may antagonize the in-vitro antitumour effect of the combination between RTX and 5FU [41]. We, however, used different cell models and a different approach in our study. In addition, a paper by Longo *et al.* [27] reported (as data not shown) the ability of FA to increase the synergism of RTX/5FU combination treatment *in vitro*.

Furthermore, although we observed in three out of four cell lines synergistic interaction between RTX and 5FU–FA at equipotent doses (50 : 50 cytotoxic ratio), we have shown a trend of better results (lowest CI values with the best PFs by FA) using lower doses of RTX in combination with 5FU–FA (25 : 75 cytotoxic ratio). Although we are further trying to explain the molecular mechanism for this observation, a potential explanation that can explain, at least in part, our observation is based on the conclusions of the paper by van der Wilt *et al.* [42], which demonstrated how antifolates, such as RTX, enhanced the binding of FdUMP to TS, especially at low antifolate concentrations. Interestingly, a previous report by Cao *et al.* [43] showed that RTX, unlike 5FU, may obtain an optimal antitumour activity in HNSCC xenograft model also at doses lower than the maximum tolerated dose.

We suggested that the synergistic interaction between RTX and 5FU–FA is dependent on both a specific perturbation of the cell cycle kinetics and a; potentiation of apoptotic cell death. In fact, in all the cell lines we have shown that the RTX/5FU–FA combination induced a slight increase with a different shape of the S-phase block in comparison with that observed in cells treated with 5FU–FA or RTX/5FU combination. Moreover, a statistical significant potentiation of apoptosis induced by RTX/5FU–FA compared with RTX/5FU or 5FU–FA has been demonstrated in all four cell lines.

In our cell models no correlation was found between p53 status or TS expression and the IC_{50} values for either RTX or 5FU–FA; however, a different pattern of growth inhibitory effect between these two agents was observed. In addition, we have shown the formation of the inactive ternary complex among TS, FdUMP and CH_2THF upon RTX/5FU–FA, suggesting that RTX does not affect the biochemical inhibition of TS by 5FU–FA.

We hypothesized that the distinct mechanism of action of RTX compared with 5FU–FA may explain the synergistic antitumour effect we have observed. It is well documented that both RTX and 5FU induce a cell cycle perturbation with an accumulation of cells in S phase [26,44]. In detail, it has been reported that RTX-induced cell cycle arrest by increasing cyclin E and cdk2 kinase activity, and in this way allows cells to progress and subsequently accumulate into S phase [45]. It has been also reported that cell cycle S-phase arrest induced by RTX, with a subsequent damage of DNA double strands, is caused by the block of DNA synthesis in the middle of replication due to dTTP depletion and not by p53-mediated G_1–G_2 checkpoint mechanisms or p21-induced inactivation of the DNA-replicating machinery [25].

In contrast, it is well known that p53 disruption makes cells strikingly resistant to the effects of 5FU, an effect that is independent from p21, and appears to be the result of perturbations in RNA, rather than DNA [23,46]. In several cases such effects of p53 mutants are attributed to dominant-negative suppression of the wt-p53 activity that results in abrogation of p53-induced apoptosis. Induction of the dUTPase gene by certain p53 mutants can additionally contribute to resistance to 5FU therapy [47]. On the contrary, RTX treatment may induce cell killing also in cells null or mutated for p53, even if the effect is reduced comparing wt-p53 cells [23–26].

In conclusion, although in our study data on the molecular mechanisms potentially affecting the pharmacological interaction between RTX and 5FU–FA are not presented, statistically significant drug interaction studies as well as the description of the distinct cell cycle perturbations leading to potentiation of apoptotic cell death are, at least at our knowledge, novel and disclose a new scenario of investigation.

The translation of the present observation in the clinical setting without considering pharmacokinetics, pharmacodynamics, rescue effects and toxicity to normal cells/tissues could be difficult. Altogether, these data may, however, have a clinical relevance because they demonstrated that FA further enhances the cytotoxic effect of RTX/5FU combination. It must be also considered that preclinical studies in mice have shown that the administration of FA 24 h after RTX may reduce its gastrointestinal toxicity [29], which has been the main life-threatening toxicity of RTX in a large multicenter randomized trial in colorectal cancer patients [48].

Therefore, our preclinical observation together with the clinical experience showing the feasibility of RTX/5FU–FA sequential schedule [34–36,49] treatment supports,

in our opinion, the further clinical investigation of these drugs in combination.

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