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ORIGINAL ARTICLE

Comparison between CoFactor and Leucovorin activity applied in combination with 5-fluorouracil against two human colon cancer cell lines

S.K. Bjelogrlić, T. Srdić, S. Radulović

Department of Experimental Pharmacology, Institute for Oncology and Radiology of Serbia, Belgrade, Serbia

Summary

Purpose: *The reduced folate leucovorin (LV) has been used for over 30 years as an enhancer of 5-fluorouracil (5-FU) antitumor activity. CoFactor (CoF) is a calcium salt of LV's active metabolite. Thus, administration of CoF can prevent decrease of intratumoral folate, which was recognized as the main reason for resistance of tumors to 5-FU treatment. The aim of this study was to investigate the cytotoxic activity and type of interactions for 5-FU and CoF applied in combination, in comparison to combination of 5-FU and LV, against human colon cancer cell lines LS-174 and HT-29.*

Methods: *The single agent activity was estimated in concentration range from 0.1 to 300 μM for 5-FU, CoF, and LV. In combined treatment each concentration of 5-FU (0.1, 1 and 10 μM) was matched with each concentration of CoF or LV (1, 10 and 100 μM). In both experiments the sulforodamine B cytotoxic test was used to assess drugs' activity after 72 h of incubation with cells. The type of interactions in combinations was calculated with the isobole method.*

Results: *5-FU showed similar activity against LS-174 and HT-29 cell lines (IC50=30.8 and 21.6 μM, respectively). While LV affected both cell lines in a slightly proliferative manner, CoF showed cytotoxic effect on both cell lines, being more active against HT-29 cell (IC50=25.7 μM) and less active on LS-174 cells (IC50=72.6 μM). Addition of LV did not change the cytotoxicity of 5-FU, especially against LS-174 cells, and synergism was found only in HT-29 cells when LV was applied in high concentrations (10 and 100 μM). However, the combination of 5-FU with CoF revealed synergistic and additive interactions in LS-174 and HT-29 cells, respectively.*

Conclusion: *These results should be taken into consideration when planning or analyzing the results of clinical trials using combination of CoF and 5-FU.*

Key words: CoFactor, colon adenocarcinoma, 5-fluorouracil, leucovorin

Introduction

The main 5-FU mode of action is irreversible inhibition of the enzyme thymidylate synthetase (TS), and hence inhibition of DNA synthesis through deoxythymidine triphosphate (dTTP) deprivation. During normal processes (Figure 1), TS forms a noncovalent ternary complex with deoxyuridine monophosphate (dUMP) and the reduced folate cofactor 5,10-methylenetetrahydrofolate (5,10-CH2-THF), catalyzing the transfer of the methylene group from 5,10-CH2-THF to dUMP to form deoxythymidine monophosphate (dTMP). The reduced folate facilitates the association and disassociation of the complex and the formation of dTMP and dihydrofolate (DHF).

The cytotoxic effects of 5-FU occur following its conversion (through the *de novo* pyrimidine pathway) to 5-fluorodeoxyuridine monophosphate (5-FdUMP). 5-FdUMP inhibits TS activity as it forms a stable inactivating ternary complex with the enzyme and 5,10-CH2-THF [1], represented on Figure 1 in the lower circle. The binding of FdUMP to TS is dependent on the intracellular concentration of 5,10-CH2-THF. Low 5,10-CH2-THF levels increase the ternary complex dissociation rate, leading to incomplete TS inhibition

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Author and address for correspondence:

Sinisa Radulovic, MD, PhD
Institute for Oncology and Radiology of Serbia
Pasterova 14
11000 Belgrade
Serbia
Tel: +381 11 2067 434
Fax: +381 11 2067 294
E-mail: sinisar@ncrc.ac.yu

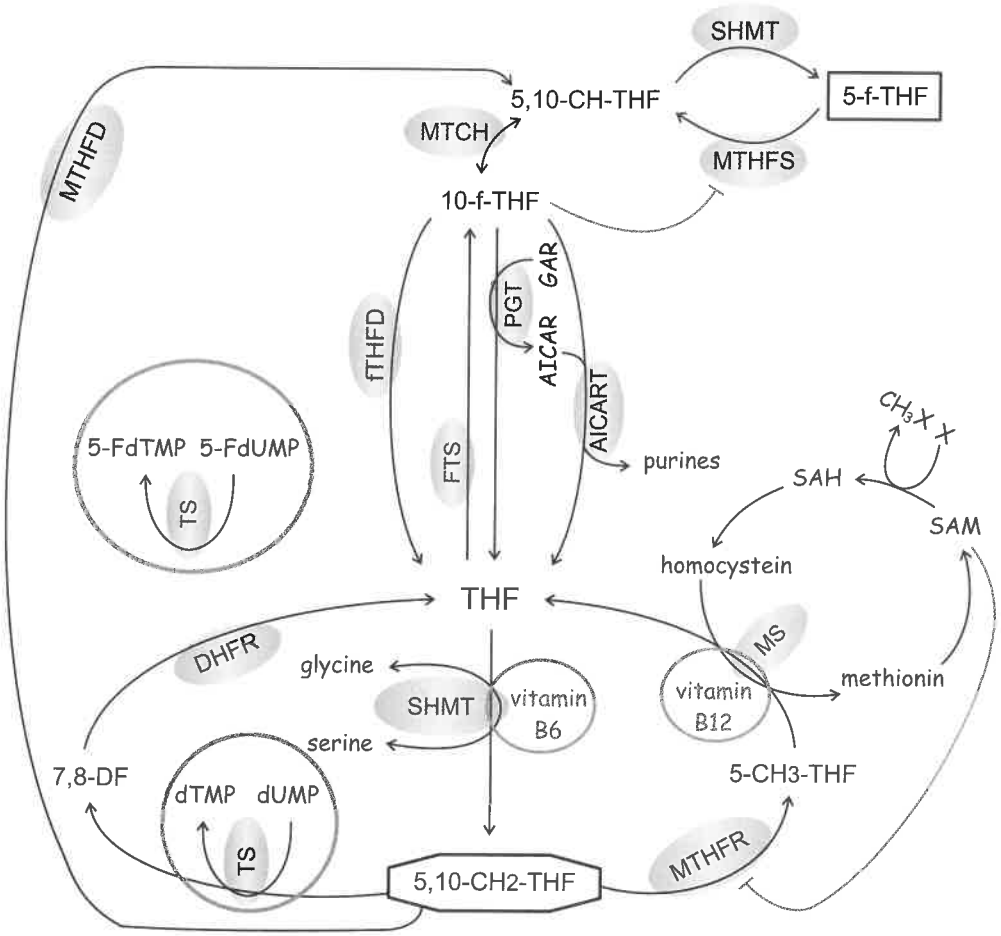


Figure 1. The folate cycle. Leucovorin (LV) and CoFactor (CoF) are represented as tetragon and octagon boxes, respectively. Enzymes are shown in ellipses. 5-f-THF: 5-formyltetrahydrogolate; SHMT: serinehydroxymethyltransferase; MTHFS: methenyltetrahydrofolate synthetase; 10-fTHF: 10-formyltetrahydrofolate; fTHFD: 10-formyltetrahydrofolate dehydrogenase; FTS: 10-formyltetrahydrofolate synthetase; PGT: phosphoribosyl glycinamidetransformylase; GAR: glycinamide ribotide; AICAR: aminoimidazolecarboxamide ribotide; AICART: aminoimidazolecarboxamide ribotide transformylase; THF: tetrahydrofolate; 5,10-CH2-THF: 5,10-methylenetetrahydrofolate; MTHFR: 5,10-methylenetetrahydrofolate reductase; 5-CH3-THF: 5-methyltetrahydrofolate; MS: methionine synthetase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; X: a variety of substrates for mutilation; TS: thymidilate synthetase; dUMP: deoxyuridine monophosphate; dTMP: deoxythimidine monophosphate; 7,8-DF: 7,8-dihydrofolate; DHFR: 7,8-dihydrofolate reductase; 5-FdUMP: 5-fluorodeoxyuridine monophosphate; 5-FdTMP: 5-fluorodeoxythymidine monophosphate; MTHFD: 5,10-methylenetetrahydrofolate dehydrogenase.

and can be responsible for decreased 5-FU activity [2]. For over two decades LV, which is racemic mixture of 5-formyltetrahydrofolate (5-f-THF), has been used as an external folate source to biomodulate (i.e. increase) the efficacy of 5-FU in the treatment of colon cancer. LV's mechanism of action is based on its entering into the folate cycle and transformation to 5,10-CH2-THF, under control of intrinsic feedback metabolic regulation processes.

The investigated drug, CoF, is the calcium salt of (6R,S) 5,10-methylene-tetrahydrofolic acid (5,10-CH2-THF). The use of CoF instead of LV (octagon and tetragon boxes on Figure 1, respectively) manages a skip from LV metabolic transformation and assure of 5,10-CH2-THF in sufficient quantum directly at the site of the action.

The aim of this study was to investigate the cytotoxic activity and type of interaction for 5-FU and CoF in combination, in comparison to combination of 5-FU and LV, against human colon carcinoma cell lines LS174-T and HT-29.

Materials and methods

Human colon adenocarcinoma cell lines HT-29 and LS174T were seeded at a density of 2500 cells/well in 96-well plates using appropriate medium (RPMI-1640 and McCoy purchased from Sigma-Aldrich) enriched with 10% fetal bovine serum (FBS). For investigation of the single agent activity, drugs were added in ascending concentrations as follows:

5-FU (Ebewe) 0.1, 1, 10, 30, and 100 μM; LV (Pfizer) 0.1, 1, 10, 100, and 300 μM; CoF (Adventrx Pharmaceuticals Inc.) 0.1, 1, 10, 30, 65, 100, and 300 μM. For investigation of the combination activity, each concentration of 5-FU (0.1, 1, and 10 μM) was matched with each concentration of LV (1, 10, and 100 μM), or CoF (1, 10, and 100 μM). Combinations were carried out as simultaneous incubations with both drugs. The cytotoxic activity (effect) of 5-FU, LV, CoF, and combinations was evaluated after 72 h of incubation by the sulforodamine B (SRB) colorimetric assay [3]. Optical densities were computed as mean (SD) of 5 replicates and the effect was calculated as:

$$\text{Effect} = E_{max} - [(OD_c - OD_b)]/[(OD_t - OD_b)]$$

where:

E_{max} – maximal possible effect to reach (equals 1)
OD_c – optical density of non-treated control cells
OD_b – optical density of blank (no cells seeded)
OD_t – optical density of treated cells

Dose-response curves, which express the relation between applied concentration and gained effect, as well as IC50 concentrations (concentration of investigated drug that declines the number of viable cells by 50% in treated cell population compared to non-treated control), were made up with GraphPad Prism software. The type of interaction (combination index, CI) between drugs in combination was assessed by the isobole method [4], and characterized as follows:

CI > 1.2 antagonism
CI = 0.8-1.2 additive interaction
CI < 0.8 synergism

Results

Single drug activity

Dose response curves and IC50 concentrations for 5-FU, CoF, and LV are presented on Figure 2. LV was the only agent which did not reveal cytotoxic but slightly proliferative activity in a dose-dependent manner on both cell lines (Figure 2c). The IC50 concentrations of 5-FU (Figure 2a, Table 1) were similar on LS174 and HT-29 cells (30.8 μM and 21.6 μM, respectively). The dose-response curves showed gradual rise of 5-FU activity in a wide range of concentrations, especially on HT-29 cells (Figure 2a), with no well defined plateau effect even at the highest concentration applied (100 μM). Unexpectedly, CoF demonstrated strong cytotoxicity against both human colon cancer cell lines (Figure 2b). As presented on Table 1, CoF was almost equally active against HT-29 cells as 5-FU (IC50 = 25.7 μM vs. 21.6 μM, respectively), but twice less active compared to 5-FU against LS174 cells (IC50 = 72.6 μM vs. 30.8 μM for 5-FU). Also, the dose-response curves of CoF action indicated its cytotoxicity in narrow concentration range. For example, on HT-29 cells CoF reached IC50 at 25.75 μM, while IC75 was already at 47.2 μM (Figure 2b, Table 1). The concentration gap between the same two concentration points was narrow on LS174 cells, too.

Table 1. The IC50 and IC75 concentrations for 5-fluorouracil and CoFactor, applied as single agents in LS174 and HT-29 cells

	5-FU (μM)		*CoF (μM)*	
	IC50	*IC75*	*IC50*	*IC75*
LS174 cells	30.8	n.d.*	72.6	90.3
HT-29 cells	21.6	n.d.*	25.7	47.2

* not defined in the applied range of concentrations

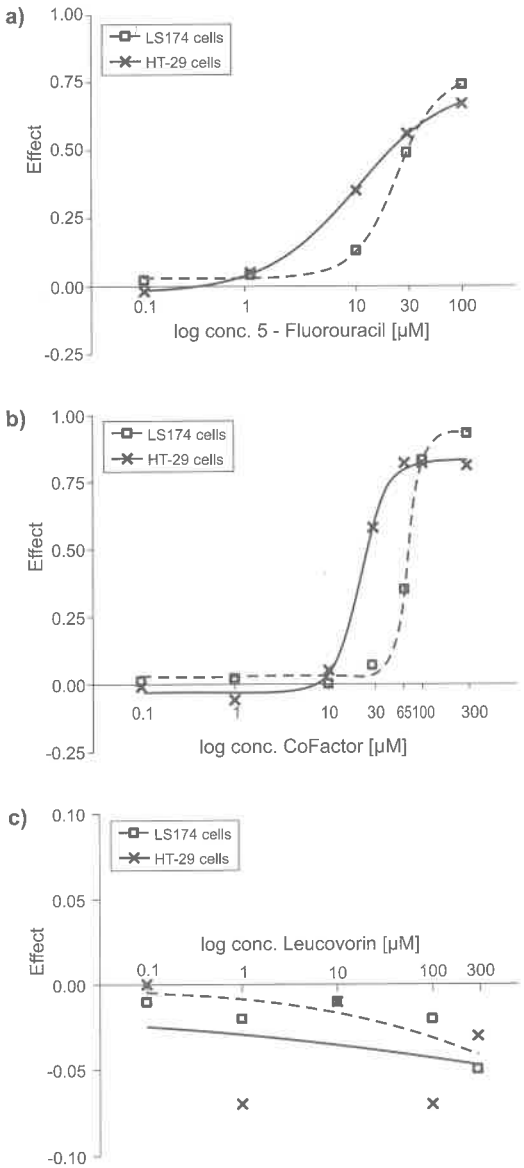


Figure 2. Dose-response curves for 5-FU (a), CoF (b), and LV (c) cytotoxic activity against LS174 and HT-29 cell lines.

Activity of drugs in combinations

LS174 cell line

The addition of LV to 5-FU did not cause significant change in cytotoxicity compared to cytotoxicity of 5-FU alone (Figure 3a). Synergistic interactions were defined in combinations with low LV concentration, but with the dose escalation of both drugs, their interactions became antagonistic (Figure 3b).

When CoF was added to 5-FU, considerable increase in cytotoxic activity was achieved compared to single-drug effects (Figure 3c vs. Figures 2a and 2b). The greatest potentiation in the activity of 5-FU was attained in combinations with the lowest CoF concentration, which correlates with 5-FU dose (Figure 3c). Even when CoF was applied in concentration of 100 μM, which is far above its IC50 obtained on LS174 cells, it further potentiated the activity of 5-FU. Significant synergism for these two drugs applied together on LS174 cells was found for all proportions of 5-FU and CoF concentrations tested (Figure 3d).

HT-29 cell line

When 5-FU was combined with LV, irrespectively of the applied concentration of LV, the resulting cytotoxic activity was similar to the cytotoxic activity obtained by 5-FU alone (Figure 4a). The type of interaction in combinations reflected an inverse 5-FU and LV concentration relationship compared to LS174 cells e.g. as the concentration of both drugs in combination got higher the interaction inclined to synergism (Figure 4b).

Even though HT-29 cell line was designated as more sensitive than LS174 cells to 5-FU and CoF in single exposure (Figure 2), application of these two drugs in combination resulted in significantly lower cytotoxic activity compared to that achieved on LS174 cell line (Figure 4c vs. Figure 3c). Synergism was defined in combination with the lowest concentrations of 5-FU and CoF (Figure 4d). Only the highest concentration of CoF gave additive interaction with every concentration of 5-FU; also, no more than the maximum dose of 5-FU evoked the same outcome with each concentration of CoF (Figure 4d).

Discussion

Fluorouracil inhibits TS through the covalent binding of FdUMP and 5,10-CH2-THF. The mecha-

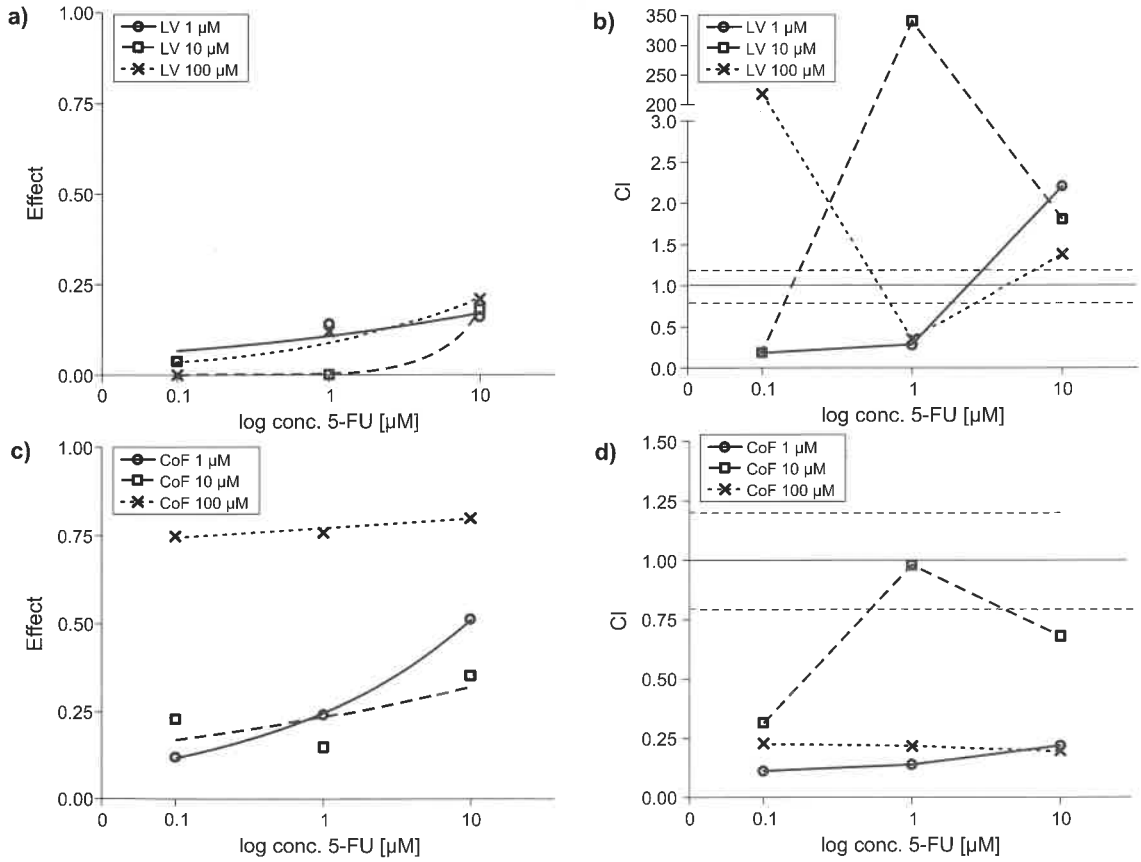


Figure 3. Dose-effect curves (a, c) and combination index graphs (b, d) for 5-fluorouracil in combination with leucovorin (a, b) or CoFactor (c, d) in LS174 cell line.

nism of resistance to the drug have been directly examined in tumor specimens of patients treated with 5-FU [5,6]. These studies confirmed the central role played by TS inhibition in 5-FU antitumor activity and underline the fact that low intracellular 5,10-CH2-THF level is probably the only reparable parameter associated with clinically impaired 5-FU activity.

For over 20 years LV has been the only additional source of folate cofactors. LV is a racemic mixture of the diasteroisomers of the 5-formyl derivative of tetrahydrofolic acid [(d,l)-5-f-THF]. Its use relied on the conviction that the l-isomer of LV is rapidly and completely metabolized to other reduced folates including 5,10-CH2-THF [7]. Recent investigations revealed that 5-f-THF is the only folate derivative that does not serve as a cofactor in folate-dependent one-carbon metabolism. It has been proposed that 1) it serves as a storage form of folate because it is chemically stable and 2) it regulates folate-dependent one-carbon metabolism by inhibiting folate-dependent enzymes, specifically targeting folate-dependent *de novo* purine biosynthesis [8]. Methenyltetrahydrofolate synthetase (MTHFS) is the only enzyme that metabolizes 5-f-THF and catalyzes its ATP-dependent conversion to 5,10-methenyltetrahydrofolate (5,10-CH-THF). Steady-state kinetic studies revealed that 10-formyltetrahydrofolate (10-f-THF), which exists in chemical equilibrium with 5,10-CH-THF, acts as a tight binding inhibitor of mouse MTHFS. Feedback metabolic regulation of MTHFS by 10-f-THF indicates that 5-f-THF can only accumulate in the presence of 10-f-THF, providing the first evidence that 5-f-THF is a storage form of excess formylated folates in mammalian cells [8].

The aim of this study was to compare the activity of 5-FU with CoF in combination against 5-FU plus LV. Examination of the single-agent activity for 5-FU, LV, and CoF was performed as part of the standard procedure. Since CoF is a downstream product of LV metabolism, it was not expected to achieve cytotoxic effect when applied alone. Instead, CoF revealed activity against HT-29 cells with IC50 concentration very close to that of 5-FU (25.7 vs. 21.6 μM, respectively). When applied on LS174 cell line, CoF was less effective compared to 5-FU, but its IC50 concentration was still below 100 μM. Also, dose-response curves for CoF had much steeper exponential phase compared

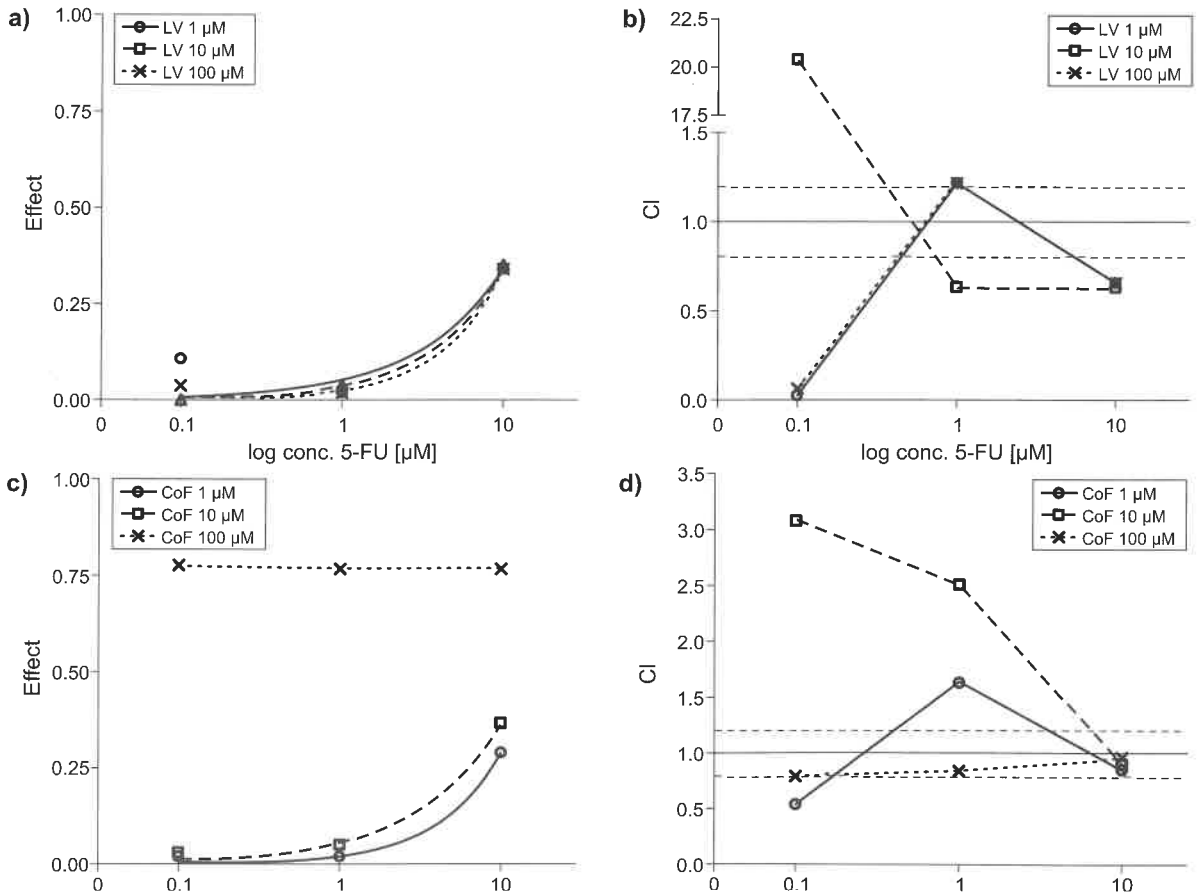


Figure 4. Dose-effect curves (a, c) and combination index graphs (b, d) for 5-fluorouracil in combination with leucovorin (a, b) or CoFactor (c, d) in HT-29 cell line.

to 5-FU, indicating that CoF is acting in tight concentration range on both HT-29 (10-65 μM) and LS174 (30-100 μM) cell lines (Figure 2b).

Combination of 5-FU with LV did not result in considerable increase of cytotoxic activity compared to the effect of 5-FU as single agent. In this situation it is not of vital interest to define the type of interaction between drugs. Anyway, when CI values for different concentrations of 5-FU and LV in combination were plotted, they gave a scattered picture of 5-FU and LV mutual relations. It is hard to establish a principle interaction pattern in regard to different concentrations ratios of these two drugs in combination. Generally, dose escalation of both drugs in combination led to antagonism on LS174 cell line, while on HT-29 cells the same dose manipulation resulted in synergistic interaction.

The addition of CoF to 5-FU gave different outcome compared to the combination of 5-FU and LV. The cytotoxic activity of 5-FU was significantly enhanced on LS174 cells, determined as synergistic interaction, especially in combinations with highest and lowest CoF concentration. Both drugs as single agents revealed to be more active against HT-29 than LS174 cells, but their combination applied to HT-29 cells did not induce such strong effect as on LS174 cell line. This difference of the combination activity on two colon cancer cell lines could be due to diverse reasons, but is probably related to variations in activity of the enzymes acting downstream of CoF (5,10-CH2-THF, octagon box on Figure 1). On the other hand, the cytotoxic activity of folate cycle intermediates has never been reported. Nevertheless, in several preclinical studies on rodent colorectal and pancreatic xenograft models, the established optimal daily dose of CoF for enhancing TS inhibition by 5-FU was 60 mg/m^2; the 120 mg/m^2 dose was associated with less TS inhibition and less antitumor effect [9-11]. These results are in inverse relation to our *in vitro* data.

The results of our investigation settles the combination of 5-FU with CoF as more effective against HT-29 and LS174 human colon cancer cell lines than the combination of 5-FU with LV. It was previously reported that the addition of CoF to 5-FU therapy resulted in 100% cure rate, compared to only 45% by 5-FU plus LV and 30% by 5-FU alone in rat colon carcinoma model of hepatic metastases [12]. In further investigations it should be necessary to determine biochemical characteristics of cell lines more and less sensitive to CoF applied as single agent, as well as reasons for a decline in tumor response to CoF in combination with 5-FU compared to its own activity. These results should be of particular value for use in the prediction of tumor response to CoF alone and to its combination with 5-FU in clinical trials.

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