Down-regulation of thymidylate synthase expression and its steady-state mRNA by oxaliplatin in colon cancer cells

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Recently, evidence has accumulated that weekly 24-h infusion of high-dose 5-fluorouracil (5-FU) with leucovorin (LV, folinic acid) biochemical modulation may improve the response rates compared with the bolus 5-FU regimens in colorectal cancer (CRC). Combining the infusional 5-FU/LV (iFL) regimens with oxaliplatin or irinotecan is widely adopted to further improve treatment efficacy. Either oxaliplatin–iFL or irinotecan–iFL may achieve an overall response rate of more than 50% in the first-line treatment. Intriguingly, in the salvage treatment for metastatic CRC patients who had failed iFL, only oxaliplatin–iFL may achieve a response rate of about 13–25%. In contrast, oxaliplatin alone or irinotecan–iFL had a very low response rate of 5% or less. To test if the oxaliplatin may reverse the iFL-related 5-FU resistance in CRC, we used DLD-1 colon adenocarcinoma cells as the *in vitro* study model. First, we revealed that oxaliplatin and 5-FU act synergistically on DLD-1 cells by MTT cytotoxicity assay and median drug effect analysis. Second, we treated the DLD-1 cells with serial concentrations of oxaliplatin (0.1–10 μM). Oxaliplatin treatment results in down-regulation of free thymidylate synthase (TS) protein expression by Western blotting. Further, we analyzed the TS mRNA level by reverse transcription and real-time quantitative polymerase chain reaction assay. Oxaliplatin treatment results in down-regulation of the TS mRNA level up to 40% (mean ± SD of ratio to reference control = 0.60 ± 0.21, range 0.42–0.84). In this study, our data provide important information explaining the reason why the combination of oxaliplatin and 5-FU results in a better objective response in 5-FU-resistant patients than oxaliplatin alone does. Our data also suggest that TS down-regulation happens at the transcriptional level. TS modulation and down-regulation had, thus, shed light on the useful potential strategy to achieve objective responses in 5-FU-resistant CRC patients. *Anti-Cancer Drugs* 15:371–376 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:371–376

Keywords: colon cancer, oxaliplatin, thymidylate synthase

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Sponsorship: This study was supported by grants of the National Taiwan University Hospital (NTUH-91-S006), National Science Council (NSC91-2314-B-418-001), and National Health Research Institute (NHRI-CN-CA9201S), Taipei, Taiwan.

This study was presented in part to the 12th Annual Meeting of the European Cancer Conference (ECCO 12), Copenhagen, 21–25 September 2003.

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Received 20 October 2003 **Accepted** 10 January 2004

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide. About 40–50% of newly diagnosed CRCs will eventually progress to metastatic stages in which chemotherapy palliates the symptoms and extends the median survival [1]. From the 1960s to the 1990s, chemotherapy for metastatic CRC relied on 5-fluorouracil (5-FU), then improved with biochemical modulation of leucovorin (LV, folinic acid) [2–6]. Numerous attempts have been made to improve the efficacy of 5-FU and LV by modification of dose, schedule or route of administration; however, the optimal combinations of 5-FU/LV remain unclear [2–6].

Conventional doses/schedules (such as the Mayo Clinic regimen with a 5-day loading schedule—5-FU 425 mg/m^2/day and LV 20 mg/m^2/day for 5 days, every 4 weeks) had suboptimal tumor responses (response rate below 23%) and excessive toxicity [7,8]. Recently, evidence has accumulated that weekly 24-h infusion of high-dose 5-FU may improve the response rates compared with the bolus 5-FU regimens. In a randomized multicenter trial for metastatic CRC, Kohne *et al.* reported an overall response rate of 44% and a median survival time of 16 months using a weekly × 6 schedule of infusional 5-FU (2600 mg/m^2 24-h infusion) [9]. In another randomized study for advanced CRC, de Gramont *et al.* reported a significantly better outcome in patients treated by a similar schedule which combined 'bolus plus infusional' 5-FU compared to 'bolus' 5-FU [10]. These results suggest that 24- or 48-h infusion of high-dose 5-FU is more effective than the conventional bolus schedules.

To further improve the treatment response for metastatic CRC, combining 5-FU/LV with novel anti-cancer agents (such as oxaliplatin, irinotecan) is used [11–13]. Among

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these approaches, the infusional 5-FU/LV (iFL) regimens, such as the HDFL regimen [14], de Gramont regimen [10,15], German AIO regimen [16], etc., were widely adopted due to improved efficacy and better toxicity profiles. In the first-line treatment, either oxaliplatin–iFL (such as FOLFOX, etc.) [11,17–19] or irinotecan–iFL (such as FOLFIRI, etc.) [13,18–20] may achieve an overall response rate of more than 50%.

Intriguingly, in the salvage treatment settings (second, third line, etc.) for metastatic CRC patients who had failed 5-FU/LV (especially the iFL regimen), only oxaliplatin plus iFL (oxaliplatin–iFL) may achieve a response rate of about 13–25% [21,22]. In contrast, oxaliplatin alone or irinotecan–iFL (irinotecan plus iFL) had a very low response rate (about 5% or less) [18,19].

We previously reported that overexpression of thymidylate synthase (TS), the target enzyme of 5-FU, may predict drug resistance to HDFL in gastric cancer [23]. Moreover, our prior studies in gastric cancers showed that paclitaxel followed by HDFL has sequence-dependent synergistic cytotoxicity in TS-overexpressing cells and reverses the drug resistance via TS down-regulation [24]. We hypothesize that oxaliplatin may reverse the iFL-related 5-FU-resistance in colon cancer cells, at least partly, via modulation of TS expression or via other unknown signaling pathway(s).

Materials and methods

Cell culture of human colon cancer cells

The human colon cancer cell line, DLD-1, was obtained from the ATCC (Manassas, VA), and routinely cultured with 90% RPMI and 10% fetal calf serum. Freeze-dried powder of oxaliplatin was obtained from Sanofi-Synthelabo Research (Bagneux, France). Standard powder of 5-FU was purchased from Sigma (St Louis, MO).

MTT colorimetric assay for cytotoxicity effects of oxaliplatin and 5-FU on DLD-1

Cytotoxicity effects and IC_{50} (cytotoxic concentration of 50% cells) of oxaliplatin and 5-FU were determined by the MTT colorimetric assay.

The growth curve of the DLD-1 cells was determined first in a 96-well culture plate with various numbers of cells, ranging from 1000 to 15 000 cells/well. Based on the growth curve, we plated 5000 cells into each well that presumably will grow exponentially in 72 h. The cells were allowed to grow and stabilize overnight. Subsequently, the cells were treated with serial concentrations of oxaliplatin and 5-FU. Each treatment was performed in triplicate. After treatment, cells were washed twice with $1 \times$ PBS and then 100 µl fresh medium was added. Cells were allowed to grow for a total of 72 h after the start of drug treatment. At the end of indicated incubation time, viable cells were determined by the MTT assay; 50 µl of MTT reagent (2 mg/ml medium) was added to each well and incubated in 37°C for 3 h. The plate was centrifuged at 2000 r.p.m. for 4 min at 4°C. Medium was suctioned out and 200 µl of DMSO was added to each well. The absorbance of each well was read by a spectrophotometric plate reader at a wavelength of 540 nm.

Median drug effect analysis

For median drug effect analysis of the two-drug combination between oxaliplatin and 5-FU, we used a fixed ratio, determined by the molar concentrations of IC_{50} between 5-FU and oxaliplatin, of serial concentrations between 5-FU and oxaliplatin for the MTT assay. The synergistic, additive or antagonistic effect of the two-drug combination was analyzed with the median drug effect analysis using a computer program (CalcuSyn, version 1.1.1) [25,26]. A combination index (CI) less than 1.0 was defined as *synergism*, equal to 1.0 was defined as *additive* and greater than 1.0 was defined as *antagonism*. Two treatment schedules were tested, either oxaliplatin and 5-FU were added together for 72 h or oxaliplatin was added first for 24 h, then oxaliplatin and 5-FU were added together for an additional 48 h.

Down-regulation of TS protein expression by oxaliplatin

The expression of TS protein was determined by oxaliplatin treatment and Western blotting using a monoclonal antibody for human TS.

Dose effects of oxaliplatin on TS protein expression

We cultured the DLD-1 cells by splitting and plating 1.5×10^6 cells per 10-cm dish for collection at 24 h after the start of oxaliplatin treatment. After the cells were plated and cultured overnight, we treated them with serial of concentrations of oxaliplatin: 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 µM. In addition, we treated the cells with 10.0 µM of 5-FU as TS ternary complex control. After exposure of oxaliplatin for 24 h, we discarded the medium and washed cells twice with $1 \times$ PBS. The cells were scraped down by a cell scraper and collected by centrifuging at 1000 r.p.m. for 5 min.

Western blot analysis for TS protein expression

Total cellular protein from DLD-1 cells was extracted by lysing the cells at 4°C with lysis buffer [50 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 30 mM tetrasodium pyrophosphate, 50 mM NaF, 0.1 mM sodium orthovanadate, 50 µM, leupeptin, 50 µg/ml aprotinin and 1 mM phenylmethylsulfonylfluoride (PMSF)]. The cell lysate was centrifuged at 14 000 r.p.m. for 3 min to deplete the cell debris. Protein concentrations of the supernatants were determined by the Bio-Rad protein assay reagent and the resulting absorbance was checked by a Beckman DU-600 spectrophotometer at the wavelength a 595 nm.

Aliquots of 240 μg of each protein sample were mixed with an equal volume of 2 × sample buffer and boiled at 95°C for 5 min. Then the protein was resolved by 12.0% SDS–PAGE according to the method of Laemmli [27].

The gels were electroblotted onto a PVDF membrane, incubated with blocking solution [3% (w/v) skim milk in TBST (20 mM Tris–HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.5] for 2 h, and then incubated with the TS106 monoclonal antibody at 1:1000 dilution (1 μg/ml) (purchased from Chemicon, Temecula, CA) at 4°C overnight. In addition, we probed the blot with β-actin antibody as an internal control for the assurance of equal loading of protein in each lane.

After the overnight incubation, we washed the membrane with TBST for 5 min, 3 times. A horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin was then added as the secondary antibody at 1:5000 dilution and incubated for 1 h at room temperature. We washed the membrane with TBST for 5 min, 3 times. The protein bands were then visualized by ECL assay reagents (Amersham, Little Chalfont, UK).

Down-regulation of TS steady-state mRNA by oxaliplatin

The steady-state mRNA levels of TS after oxaliplatin treatment were determined by RNA extraction, purification, reverse transcription and real-time PCR for TS and G6PDH using the LightCycler (Roche, Mannheim, Germany) and LightCycler relative quantification software.

RNA extraction and purification

After DLD-1 cells were plated and cultured overnight, we treated them with serial of concentrations of oxaliplatin: 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μM of oxaliplatin. After exposure to oxaliplatin for 24 h, we collected the cells by the methods described above for further RNA extraction.

Total cellular RNA was prepared by acid guanidinium thiocyanate:phenol:chloroform extraction, which was based on the method of Chomczynski and Sacchi [28]. DLD-1 cells treated with each protocol were used for the RNA extraction. Cells were lysed directly in a culture dish by adding 1 ml of TRIzol RNA extraction reagent (Invitrogen, Carlsbad, CA). Cell lysate was transferred into polypropylene tubes and 0.8 ml of chloroform:isoamylalcohol mixture (24:1) was added to accelerate the separation of two layers after acid phenol extraction (pH 4.0). The upper layer which contains RNA was precipitated by isopropanol and the RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water.

Real-time quantitative RT-PCR for mRNA of TS and G6PDH using the LightCycler

The cDNA was reverse transcribed from total RNA using AMV reverse transcription and random hexamer priming.

A 111-bp fragment of TS mRNA was amplified from the cDNA by PCR in the LightCycler using specific primers. The amplicon was detected by fluorescence using a specific pair of hybridization probes. Using the same cDNA preparation, but in a separate PCR reaction, G6PDH was amplified as a reference gene. The reaction product of G6PDH served as a reference for relative quantitation and in addition as a control for RNA integrity. A calibrator RNA was included to allow for relative quantitation. All of the above studies were performed in triplicate.

Results

IC_{50} of oxaliplatin and 5-FU on DLD-1 cells

The IC_{50} (cytotoxic concentration of 50% cells) of 5-FU and oxaliplatin on DLD-1 cells was 16.7 ± 2.4 and 8.0 ± 0.2 μM (mean ± SD), respectively, which was determined by MTT colorimetric assay and calculated by the CalcuSyn computer program, version 1.1.1. All of the experiments were performed in triplicate. For further median effect analysis of the two-drug combination between oxaliplatin and 5-FU, we used a fixed ratio of serial molar concentrations between 5-FU and oxaliplatin (the ratio of 5-FU:oxaliplatin was 2:1) for the MTT assay and median drug effect analysis.

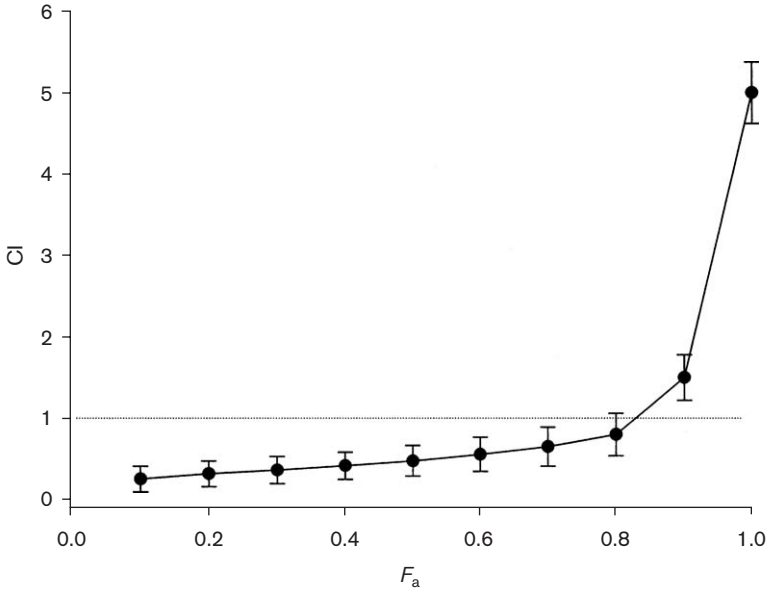
Oxaliplatin and 5-FU act synergistically on DLD-1 cells

We revealed that oxaliplatin and 5-FU act synergistically on DLD-1 cells by the MTT assay and median drug effect analysis, calculated by a CalcuSyn computer program, version 1.1.1 (Fig. 1). Two treatment schedules were tested, either oxaliplatin and 5-FU were added together for 72 h or oxaliplatin was added first for 24 h, then oxaliplatin and 5-FU were added together for an additional 48 h. Both schedules showed *synergistic* effects ($CI < 1.0$) between oxaliplatin and 5-FU at the fraction affected (F_a) at least up to 0.8. Figure 1 shows the different CIs at a series of fractions affected, using the treatment schedule of oxaliplatin and 5-FU added together for 72 h. Median drug effect analysis of another treatment schedule with similar results is not shown.

Down-regulation of free TS protein expression by oxaliplatin

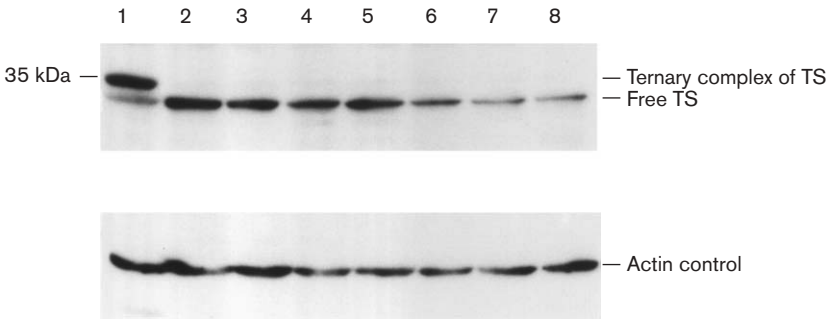
We treated the DLD-1 cells with a serial concentrations of oxaliplatin: 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μM. We used DLD-1 cells treated with 5-FU alone (at a concentration of 10.0 μM) as the control lane in these experiments; it showed both TS ternary complex (upper band) and free TS (lower band) by Western blotting using the monoclonal antibody TS106, which is specific for human TS. The oxaliplatin treatment (2.0, 5.0 and 10.0) resulted in

Fig. 1



Oxaliplatin and 5-FU act synergistically on DLD-1 cells shown by median drug effect analysis. Synergistic effects (CI < 1.0) have been shown between oxaliplatin and 5-FU at the F_a at least up to 0.8. CI denotes combination index. CI < 1.0 was defined as *synergism*, CI = 1.0 was defined as *additive* and CI > 1.0 was defined as *antagonism*.

Fig. 2



Down-regulation of free TS protein expression by oxaliplatin. We treated the DLD-1 cells without oxaliplatin (lane 2), 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μM of oxaliplatin (lanes 3–8, respectively). In addition, we treated the cells with 10.0 μM of 5-FU as a TS *ternary complex* control (lane 1). Being treated with 5-FU (10.0 μM), DLD-1 cells had both TS ternary complexes (*upper band*) and free TS (*lower band*) by Western blotting using the monoclonal antibody TS106. Oxalipaltin treatment (2.0, 5.0 and 10.0 μM) resulted in down-regulation of *free* TS protein expression (lanes 6–8, respectively).

significant down-regulation of free TS (active form) protein expression by Western blotting using the monoclonal antibody TS106 (Fig. 2).

Down-regulation of TS steady-state mRNA by oxaliplatin

We treated the DLD-1 cells with a serial concentrations of oxaliplatin: 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 μM. The oxaliplatin treatment resulted in significant down-regulation of the TS mRNA level up to 40% (mean ± SD of ratio to reference control = 0.60 ± 0.21, range 0.42–0.84) at the oxaliplatin concentration of 10.0 μM by real-time quantitative RT-PCR assay using the LightCycler and its relative quantitation software (Table 1).

Discussion

TS is the target enzyme of 5-FU [29,30]. The stabilization of a *ternary complex* among TS, reduced folates (5,10-methylene tetrahydrofolate) and FdUMP (5-fluorodeoxyuridine monophosphate) can enhance the inhibition of TS. The 'ternary complex' is an *inactive* form of TS [31,32], while the 'free' form of TS is an *active* enzyme

Table 1 Down-regulation of TS mRNA by relative quantitative analysis with real-time PCR

	Concentration of oxaliplatin (μM)						
	0	0.1	0.5	1.0	2.0	5.0	10.0
Expression ratio (mean ratio ± SD)[a]	1	0.84 ± 0.18	0.83 ± 0.09	0.70 ± 0.20	0.65 ± 0.15	0.64 ± 0.13	0.60 ± 0.21

[a] *Treated* with oxaliplatin divided by *untreated* control.

and its expression is inversely correlated with the drug sensitivity in several human cancers [33,34]. In clinical samples, overexpression of TS has been demonstrated to be closely associated with drug resistance to 5-FU-based chemotherapy in colorectal cancers [35,36] and gastric cancers [23,36,37]. We have modified the original HDFL regimen [weekly 24-h infusion of high-dose 5-FU (2600 mg/m^2) with leucovorin (500 mg/m^2)] of Ardalan *et al.* [38] with dose reduction of leucovorin to 300 mg/m^2 and had an overall response rate of 42.9% (28–59%, 95% confidence interval) in recurrent or metastatic CRC patients [14,39]. We have clarified the underlying mechanisms for improved efficacy of HDFL by prolonged and enhanced suppression of free TS by 24-h infusion of high-dose 5-FU [40].

In this study, our data provide important information explaining the reason why combination of oxaliplatin and 5-FU results in better objective response in 5-FU-resistant patients than oxaliplatin alone does. Our data also suggest that TS down-regulation happens at the transcriptional level. Further exploration to clarify the exact mechanism(s) for the TS down-regulation at the transcriptional level is warranted. Oxaliplatin, a platinum-based chemotherapeutic agent with a non-hydrolyzable 1,2-diaminocyclohexane (DACH) carrier ligand which is maintained in the final cytotoxic metabolites of the drug, has shown *in vitro* and *in vivo* efficacy against many tumor cell lines, including some that are resistant to cisplatin and carboplatin [41]. Like cisplatin, oxaliplatin targets DNA producing mainly 1,2-GG intrastrand cross-links. The intrinsic chemical and steric characteristics of the DACH–platinum adducts appear to contribute to the lack of cross-resistance with cisplatin [42]. It is also probable that the retention of the bulky DACH ring by activated oxaliplatin can interfere with the transcription of TS.

Treating the CRC patients who had failed the iFL regimens, oxaliplatin plus iFL has a significantly higher response rate than irinotecan plus iFL, irinotecan plus oxaliplatin [43] or oxaliplatin alone [44]. Taking the clinical and preclinical evidence together, TS modulation and down-regulation by oxaliplatin or other potential agents has shed light on the useful strategies to achieve objective an response in 5-FU-resistant CRC patients.

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