Cancer Chemother Pharmacol (1991) 28: 448–454
0344570491001321

Cancer Chemotherapy and Pharmacology



# Sequence-dependent cytotoxic effects of the combination of a new nitrosourea, fotemustine, with 5-fluorouracil plus folinic acid

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Received 23 February 1991/Accepted 10 June 1991

**Summary.** The present study was designed to analyse the cytotoxic effects of the combination of fotemustine with 5-fluorouracil (5-FU) plus folinic acid (FA). Two human tumor cell lines were used; one line was derived from colon cancer (WIDR) and the other, from a non-small-cell lung cancer (CAL 12). Cytotoxic effects were assessed using the MTT (tetrazolium bromide) semi-automated test in 96-well incubation plates. The effects of various drug combinations were evaluated by the isobologram method. The drug combinations tested included fotemustine concentrations of 20, 30, 40, 50 and 70 μg/ml, 5-FU concentrations of 5, 15 and 30 μg/ml, and a constant FA concentration of $10^{-5}$ M. A total of 180 different experimental conditions were tested. When cells were exposed to fotemustine prior to treatment with 5-FU, the final cytotoxic effects on both cell lines were additive or synergistic in the majority of cases ($P < 0.001$). The 5-FU concentration was a determinant factor that modified the effects of the drug combination from antagonism (at low 5-FU concentrations) to synergism (high 5-FU concentrations; $P < 0.001$). The addition of FA ($10^{-5}$ M) resulted in a significant shift towards synergistic associations in both cell lines. Administration of 5-FU prior to treatment with fotemustine caused marked antagonism, which $10^{-5}$ M FA could not significantly shift towards simple additivity.

## Introduction

The treatment of colon carcinoma remains a major challenge in cancer chemotherapy. The response rate achieved using active drugs against tumors in this localization remains disappointing [8], and despite its low intrinsic activity, 5-fluorouracil (5-FU) may nevertheless be considered the drug of choice [11]. The cytotoxic activity of 5-FU can be potentiated by its combination with several drugs, and the bases for biochemical modulation of this drug have been well elucidated for methotrexate, dipyridamole, cisplatin, and folinic acid (FA) [10]. The association of 5-FU and FA has proved to be clinically superior to 5-FU alone for the treatment of colorectal cancer [1]. Combination of 5-FU with an alkylating agent has been found to produce synergistic cytotoxic effects in tumor-bearing mice [19]. However, in patients presenting with colorectal carcinoma, the administration of 5-FU with the nitrosourea tauromustine has produced an unexpectedly low response rate [23].

Fotemustine is a new drug that is representative of the new generation of nitrosoureas containing a special carrier group [13]. In a previous in vitro study, we demonstrated that the cytotoxic activity of fotemustine was comparable with, if not higher than, that of carmustine, which was used as a reference [12]. The present study was conducted to analyse the cytotoxic effects of a combination of fotemustine, 5-FU and FA on two human cancer cell lines. The isobologram method was used to evaluate the effects of the various drug combinations.

## Materials and methods

*Drugs.* Fotemustine was obtained from the Institut de Recherches Internationales Servier (Courbevoie, France) as a 50-mg/ml solution in 95% (v/v) ethanol. FA was obtained from Sigma (La Verpillière, France). 5-FU was used as a commercial preparation (Produits Roche, Neuilly, France). All drugs were stored at –20°C and constituted the stock solutions. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, and foetal bovine serum (FBS) were obtained from Gibco (Paisley, UK). Penicillin and streptomycin were supplied by Merieux (Lyons, France). The MTT test was performed using 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide (DMSO), both of which were obtained from Sigma.

*Cell cultures.* Two human tumor cell lines were used: WIDR, derived from colon cancer, and CAL 12, derived from a non-small-cell lung cancer. WIDR was obtained from the CASSG EORTC group, and CAL 12 was isolated at our institute. CAL 12 has a mean doubling time of 35 h and is characterised by the presence of epidermal growth factor (EGF) receptors with two families of sites ($Kd_1 = 0.051$ nM, $n = 71{,}000$/cell; $Kd_2 = 1.85$ nM, $n = 175{,}000$/cell). Cells were routinely cultured in a

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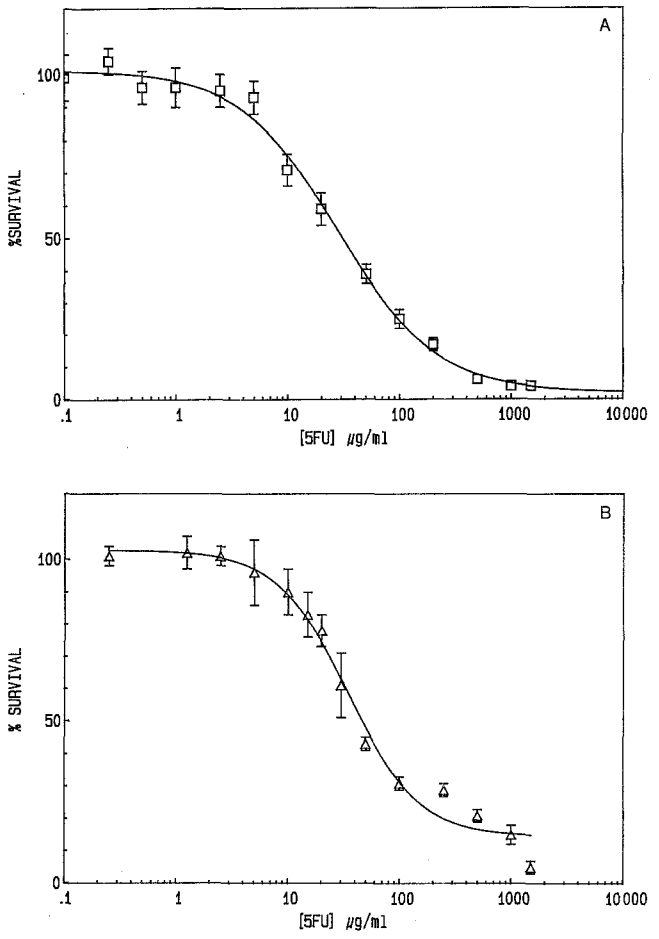


**Fig. 1 A, B.** Dose-response curves for **A** WIDR and **B** CAL 12 cells following treatment with 5-FU. Both curves were best fit by sigmoid curves, with $r^2$ values of 0.995 and 0.985 being obtained for WIDR and CAL 12 cells, respectively. *% Survival*, percentage of viable cells as compared with untreated controls

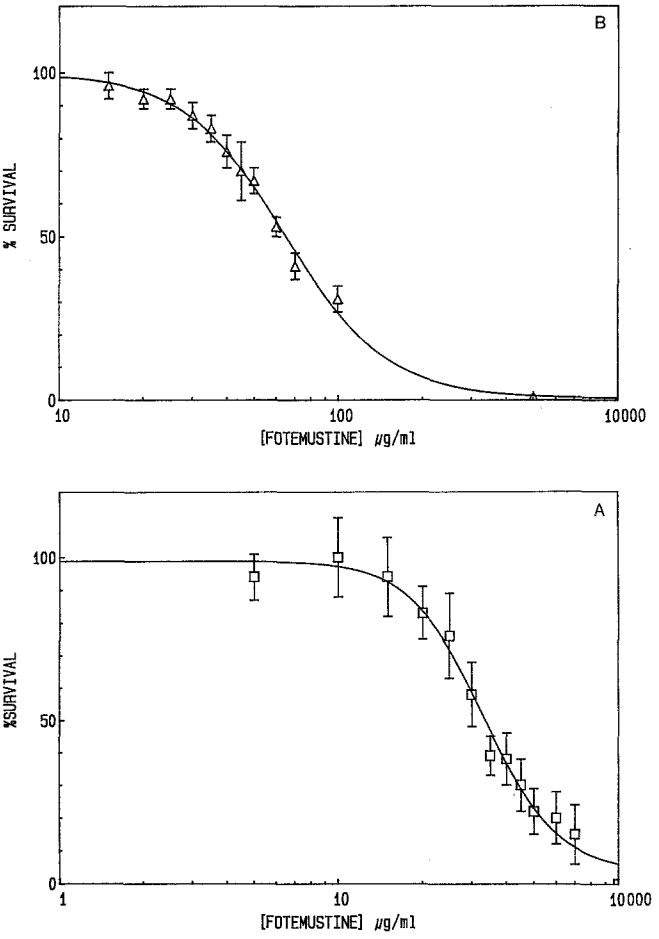


**Fig. 2 A, B.** Dose-response curves for **A** WIDR and **B** CAL 12 cells following treatment with fotemustine. Both curves were best fit by sigmoid curves, with $r^2$ values of 0.987 and 0.996 being obtained for WIDR and CAL 12 cells, respectively. *% Survival*, percentage of viable cells as compared with untreated controls

humidified incubator (Sanyo) at 37° C in an atmosphere comprising 8% $CO_2$ in air. Cells were grown in DMEM supplemented with 10% FBS, penicillin (50,000 IU $l^{-1}$), streptomycin (86 µM), and L-glutamine (2 mM). Cells were grown in 96-well microtitration plates, 5-FU concentrations ranged between 0 and 1500 µg/ml; fotemustine levels ranged between 0 and 200 µg/ml. The FA concentration was $10^{-5}$ M (5 µg/ml); this concentration was selected as the optimal FA concentration on the basis of our previous experience in studies using combinations of 5-FU, cisplatin and FA on cancer cell lines in vitro (manuscript in preparation). The duration of cell exposure to either 5-FU alone or 5-FU plus FA was 1 h. Cells were exposed to fotemustine for 2 h. Fotemustine was added either before (–24 h), during, or after (+24 h) exposure to 5-FU or 5-FU plus FA. The concentration-time products ($c \times t$) of the drugs tested were compatible with their respective clinical pharmacokinetics [15, 20, 25].

*Evaluation of cytotoxicity.* The cytotoxic effects of the drugs tested alone and in combination were assessed at 6 days after exposure using the MTT semi-automated method [5, 24] in 96-well incubation plates. Results were expressed as the relative percentage of absorbance as compared with that in untreated controls. Absorbance was set at 540 nm and was measured on a Titertek Twin reader. Each experimental point was determined in sextuplicate. For all experiments, the coefficient of variation ranged between 3% and 10%. $IC_{50}$ was defined as the concentration inhibiting 50% of the cell growth as compared with that in untreated controls. The effects of the different drug combinations were evaluated using the isobologram method [21]. In brief, the dose-effect curves determined experimentally for each drug were assimilated to a sigmoid curve:

$$y = \frac{2}{1 + \left| \frac{\chi}{C1} \right|^{-p}},$$

where $y = \log_{10}$ of the percentage of survival for a concentration $K$ of $x = \log_{10} K$; $C1$ = value of $x$ for $y = 1$; C1 = value of $x$ when the percentage of survival = 10 ; and $p$ = "Hill" slope of the sigmoid curve, whereby $CA$ is the value of $C1$ for drug $A$, $cB$ is the value of $C1$ for drug B, $D$ is the value of $p$ for drug $A$, $\delta$ is the value of $p$ for drug $B$, $ye = \log E$, $E$ = survival observed after the action of a given concentration ($a$) of drug $A$ and a given concentration ($b$) of drug $B$, $xA$ = concentration of drug $A$ and $xB$ = concentration of drug $B$.

The isobologram associated with a given effect $E$ consists of two envelope curves: mode 1 (independent mechanisms of drug action) and mode 2 (common mechanisms of drug action). Each envelope curve gives a relationship between $xA$ and $xB$:

$$\text{Mode1:} \quad xA = CA \left| \left( \frac{2-ye}{ye} \right)^{\frac{-1}{D}} - \left( \frac{\chi B}{CB} \right)^{\frac{\delta}{D}} \right|$$

$$\text{Mode 2:} \quad xA : CA \left| \frac{2}{\frac{2}{1 + \left( \frac{\chi B}{CB} \right)^{-\delta}} - ye} - 1 \right|^{1/D}.$$

**Table 1.** Cytotoxic effects of the incubation of WIDR cells with fotemustine at 24 h prior to 5-FU treatment

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA | | | With FA | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 97 | 85 | | | ■ | | | ■ |
| | 15 | 58 | 31 | | ■ | | ■ | | |
| | 30 | 29 | 10 | ■ | | | ■ | | |
| 30 | 5 | 83 | 81 | | | ■ | | | ■ |
| | 15 | 49 | 19 | | ■ | | ■ | | |
| | 30 | 24 | 10 | ■ | | | ■ | | |
| 40 | 5 | 69 | 71 | | | ■ | | | ■ |
| | 15 | 39 | 16 | | ■ | | ■ | | |
| | 30 | 18 | 6 | ■ | | | ■ | | |
| 50 | 5 | 57 | 53 | | | ■ | | | ■ |
| | 15 | 27 | 10 | | ■ | | ■ | | |
| | 30 | 15 | 5 | ■ | | | ■ | | |
| 70 | 5 | 35 | 25 | | | ■ | | ■ | |
| | 15 | 16 | 5 | | ■ | | ■ | | |
| | 30 | 11 | 4 | ■ | | | ■ | | |

Syn, Synergy; Add, additivity; Ant, antagonism

For each effect $E$, there is one isobologram. The intersection of the coordinates (a, b) may lie:

1. Above the envelope, in which case the effects of the combination of drugs $A$ and $B$ are less than additive (antagonism)
2. Within the envelope, in which case the effects of the combination of drugs $A$ and $B$ are additive (simple additivity)
3. Below the envelope, in which case the effects of the combination of drugs $A$ and $B$ are more than additive (supra-additivity or synergy)

Isobolograms were computerized using the mathematical software program Statgraphics (Statistical Graphics Corporation, Rockville, Md., USA).

## Results

Figures 1 and 2 show the dose-response curves for the two cell lines following exposure to 5-FU and fotemustine, respectively. Curves were best fit by sigmoid plots; the $IC_{50}$ values (μg/ml) for 5-FU and fotemustine, respectively, were 30 and 33 in WIDR cells and 35 and 62 in CAL 12 cells.

Tables 1–6 detail the results of isobologram analysis. The drug combinations tested included fotemustine concentrations of 20, 30, 40, 50 and 70 μg/ml, 5-FU concentrations of 5, 15 and 30 μg/ml, and a constant FA concentration of $10^{-5}$ M. A total of 180 different experimental conditions were tested and evaluated by isobolographic analysis. When cells were exposed to fotemustine prior to treatment with 5-FU, the final cytotoxic effects on both cell lines were additive or synergistic in the majority of cases ($P$ <0.001; Tables 1, 2). The 5-FU concentration was a determinant factor that modified the effects of a combination from antagonism (at low 5-FU concentrations) to synergism (high 5-FU concentrations; $P$ <0.001); this finding was observed systematically in WIDR cells (Table 1) and was seen at the lowest fotemustine concentrations in CAL 12 cells (Table 2). Addition of FA ($10^{-5}$ M) produced a significant shift towards synergistic associations in both cell lines ($P$ <0.001). Simultaneous administration of fotemustine and 5-FU (Tables 3, 4) led to antagonistic effects ($P$ <0.001), which were particularly marked in CAL 12 cells (Table 4). Addition of FA ($10^{-5}$ M) produced a change from antagonism towards additivity (CAL 12 cells, Table 4) or even synergy (WIDR cells, Table 3; $P$ <0.001). When 5-FU was used before fotemustine, we observed a marked antagonism, which $10^{-5}$ M FA failed to shift significantly towards simple additivity (Tables 5, 6).

## Discussion

Biochemical modulation is now a well-established technique in cancer chemotherapy [10]. The recently developed drug combination of 5-FU plus FA has a strong pharmacological basis [16] and has proved to be more effective than 5-FU alone for the treatment of colon cancer [1]. A synergistic interaction has been demonstrated between nitrosoureas and 5-FU in tumor-bearing mice [19]. Fotemustine, a new nitrosourea that is active against carcinomas of the colon [14] and the lung [18] exhibits an activity that is equivalent, if not superior, to that of carmustine [12]. This observation prompted us to evaluate the effects of a multiple drug combination including fotemustine, 5-FU and FA on cancer cell lines in vitro. The intrinsic limitations of such in vitro studies should be borne in mind, particularly the lack of information on the toxicity of those drugs to normal tissue. Different sequences of administration were tested, and the synergy, simple additivity or antagonism between the drugs was evaluated using an appropriate isobolographic method. A wide range of drug concentrations were tested, including those used for clinically treatment of patients [15, 20, 25].

**Table 2.** Cytotoxic effects of the incubation of CAL 12 cells with fotemustine at 24 h prior to 5-FU treatment

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA[a] | | | With FA[b] | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 98 | 87 | | | ■ | | ■ | |
| | 15 | 79 | 55 | | ■ | | ■ | | |
| | 30 | 60 | 41 | ■ | | | ■ | | |
| 30 | 5 | 95 | 89 | | | ■ | | | ■ |
| | 15 | 72 | 55 | | ■ | | ■ | | |
| | 30 | 50 | 35 | ■ | | | ■ | | |
| 40 | 5 | 73 | 63 | | ■ | | ■ | | |
| | 15 | 44 | 33 | ■ | | | ■ | | |
| | 30 | 43 | 29 | ■ | | | ■ | | |
| 50 | 5 | 64 | 59 | | ■ | | ■ | | |
| | 15 | 54 | 41 | | ■ | | ■ | | |
| | 30 | 44 | 31 | | ■ | | ■ | | |
| 70 | 5 | 45 | 37 | ■ | | | ■ | | |
| | 15 | 44 | 33 | ■ | | | ■ | | |
| | 30 | 35 | 29 | ■ | | | ■ | | |

Syn, Synergy; Add, additivity; Ant, antagonism

[a] Statistical analysis (Table 1 plus Table 2 data): there was significantly more synergy or additivity than antagonism ($X^2 = 70.53$, $P < 0.001$); moreover, there was a significant change from antagonism towards synergy with increasing 5-FU concentration (Spearman rank correlation, $P < 0.001$)

[b] Statistical analysis (Table 1 plus Table 2 data): more synergy was observed in the presence of FA than in its absence ($X^2 = 34.9$, $P < 0.001$)

**Table 3.** Cytotoxic effects of simultaneous incubation of WIDR cells with fotemustine and 5-FU

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA | | | With FA | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 98 | 101 | | | ■ | | | ■ |
| | 15 | 85 | 60 | | | ■ | | ■ | |
| | 30 | 54 | 6 | | | ■ | ■ | | |
| 30 | 5 | 96 | 87 | | | ■ | | | ■ |
| | 15 | 77 | 29 | | | ■ | | ■ | |
| | 30 | 50 | 6 | | | ■ | ■ | | |
| 40 | 5 | 91 | 81 | | | ■ | | ■ | |
| | 15 | 62 | 37 | | | ■ | | ■ | |
| | 30 | 36 | 6 | | ■ | | ■ | | |
| 50 | 5 | 80 | 67 | | | ■ | | ■ | |
| | 15 | 54 | 37 | | ■ | | | ■ | |
| | 30 | 30 | 4 | | ■ | | ■ | | |
| 70 | 5 | 61 | 46 | | | ■ | | ■ | |
| | 15 | 33 | 9 | | ■ | | | ■ | |
| | 30 | 17 | 3 | | ■ | | ■ | | |

Syn, Synergy; Add, additivity; Ant, antagonism

Our findings for both cell lines revealed that the combination of fotemustine and 5-FU produced opposite effects, depending on the sequence of drug exposure. Administration of 5-FU before fotemustine produced marked antagonism in the resulting cytotoxic effects, whereas a majority of synergistic or additive effects was observed for the opposite sequence. A similar influence of the order of drug administration has previously been demonstrated for the sequential combination of 5-FU and methotrexate. These two agents exhibited cytotoxic synergism when methotrexate was given before 5-FU [3, 4], and the reverse sequence of drug administration reportedly produced the least favorable results due to antagonism [2]. These two drugs are antimetabolites, and they act at least partially on

**Table 4.** Cytotoxic effects of simultaneous incubation of CAL 12 cells with fotemustine and 5-FU

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA[a] | | | With FA[b] | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 92 | 99 | | | ■ | | | ■ |
| | 15 | 81 | 76 | | | ■ | | | ■ |
| | 30 | 58 | 44 | | | ■ | | ■ | |
| 30 | 5 | 87 | 95 | | | ■ | | | ■ |
| | 15 | 73 | 64 | | | ■ | | ■ | |
| | 30 | 59 | 48 | | | ■ | | ■ | |
| 40 | 5 | 93 | 94 | | | ■ | | | ■ |
| | 15 | 68 | 55 | | | ■ | | ■ | |
| | 30 | 53 | 47 | | | ■ | | ■ | |
| 50 | 5 | 74 | 79 | | | ■ | | | ■ |
| | 15 | 60 | 54 | | | ■ | | ■ | |
| | 30 | 50 | 43 | | | ■ | | ■ | |
| 70 | 5 | 53 | 54 | | ■ | | | ■ | |
| | 15 | 50 | 35 | | ■ | | | ■ | |
| | 30 | 58 | 42 | | | ■ | | ■ | |

Syn, Synergy; Add, additivity; Ant, antagonism

[a] Statistical analysis (Table 3 plus Table 4 data): there was more antagonism than additivity ($X^2 = 38.5$, $P < 0.001$)

[b] Statistical analysis (Table 3 plus Table 4 data): there was more additivity or synergy than antagonism ($X^2 = 38.5$, $P < 0.001$)

**Table 5.** Cytotoxic effects of the incubation of WIDR cells with fotemustine at 24 h following treatment with 5-FU

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA | | | With FA | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 81 | 82 | | ■ | | | ■ | |
| | 15 | 93 | 81 | | | ■ | | | ■ |
| | 30 | 62 | 51 | | | ■ | | | ■ |
| 30 | 5 | 93 | 91 | | | ■ | | | ■ |
| | 15 | 85 | 76 | | | ■ | | | ■ |
| | 30 | 67 | 41 | | | ■ | | ■ | |
| 40 | 5 | 75 | 75 | | | ■ | | | ■ |
| | 15 | 71 | 60 | | | ■ | | | ■ |
| | 30 | 49 | 47 | | | ■ | | | ■ |
| 50 | 5 | 66 | 73 | | | ■ | | | ■ |
| | 15 | 74 | 59 | | | ■ | | | ■ |
| | 30 | 44 | 35 | | | ■ | | | ■ |
| 70 | 5 | 54 | 60 | | | ■ | | | ■ |
| | 15 | 43 | 46 | | | ■ | | | ■ |
| | 30 | 21 | 16 | | | ■ | | | ■ |

Syn, Synergy; Add, additivity; Ant, antagonism

common intracellular targets; the biochemical origins of sequence-dependent cytotoxic effects have been relatively well elucidated [10].

The pharmacological basis of the sequence-dependent effect observed in the present study between the nitrosourea fotemustine and 5-FU is harder to define. The cytotoxicity of 5-FU is known to involve the activated forms of the drug, which inhibit thymidilate synthase (TS) activity and, thus, DNA synthesis, and/or the direct incorporation of the drug into RNA [17]. More recently, fluorodeoxyuridine triphosphate incorporation into the DNA molecule has been identified as a possible mechanism of cytotoxicity [6]. As nitrosoureas act via an alkylating attack on DNA and proteins, more or less additive effects of both drugs on the DNA molecule itself may explain the observed effects.

The effectiveness of DNA repair following damage due to alkylation is a determinant factor in the cytotoxic effects of alkylating agents [9]. DNA repair may represent a more

**Table 6.** Cytotoxic effects of the incubation of CAL 12 cells with fotemustine at 24 h following treatment with 5-FU

| Fotemustine concentration (μg/ml) | 5-FU concentration (μg/ml) | Observed cell survival (%) | | Isobolographic analysis | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Without FA | With FA | Without FA[a] | | | With FA[a] | | |
| | | | | Syn | Add | Ant | Syn | Add | Ant |
| 20 | 5 | 92 | 106 | | | ■ | | | ■ |
| | 15 | 106 | 95 | | | ■ | | | ■ |
| | 30 | 96 | 74 | | | ■ | | | ■ |
| 30 | 5 | 90 | 103 | | | ■ | | | ■ |
| | 15 | 98 | 101 | | | ■ | | | ■ |
| | 30 | 85 | 70 | | | ■ | | | ■ |
| 40 | 5 | 86 | 93 | | | ■ | | | ■ |
| | 15 | 69 | 70 | | | ■ | | | ■ |
| | 30 | 62 | 46 | | | ■ | | ■ | |
| 50 | 5 | 75 | 71 | | | ■ | | ■ | |
| | 15 | 66 | 57 | | | ■ | | ■ | |
| | 30 | 55 | 47 | | | ■ | | ■ | |
| 70 | 5 | 54 | 63 | | ■ | | | | ■ |
| | 15 | 45 | 56 | | ■ | | | | ■ |
| | 30 | 41 | 43 | | ■ | | | ■ | |

Syn, Synergy; Add, additivity; Ant, antagonism

[a] Statistical analysis (Table 5 plus Table 6 data): there was a significant preponderance of antagonism ($X^2 = 101.6$, $P < 0.001$) in the presence and absence of FA

realistic explanation for the characteristic effects caused by the present drug combination. Inhibition of TS by 5-FU may result in a lack of appropriate nucleotide precursors for the repair of damage due to alkylation. This biochemical hypothesis concurs with the synergistic toxic effects that were observed when fotemustine was used before 5-FU. The enhancing effect of FA is also compatible with this hypothesis, as FA amplifies the action of 5-FU by increasing the inhibition of TS [16].

However, the 5-FU concentration was a significant factor that determined the final expression of cytotoxicity when fotemustine was used prior to 5-FU. This was especially true for WIDR cells. Because TS activity is partially blocked by low 5-FU concentrations, the natural substrate deoxyuridine monophosphate accumulates behind the enzymatic block, leading to substrate competition with the 5-FU-induced inhibition, a reduction in 5-FU-mediated effects, and repletion of DNA precursors that are necessary for the repair of damage due to alkylation. This may explain why a preponderance of antagonistic effects were observed at low 5-FU concentrations when the sequence of fotemustine followed by 5-FU was used. A rebound in TS activity following 5-FU exposure has been described in experimental models [7] and in human carcinomas treated with 5-FU [22]. This rise in TS activity leads to an increase in DNA precursors, which favor the repair of alkylation damage. This may explain why the sequence of 5-FU followed by fotemustine produced antagonistic cytotoxic effects. Other underlying biochemical pathways may also be involved.

Overall, the combination of fotemustine and 5-FU plus FA exhibited sequence-dependent effects; use of the appropriate drug sequence and drug doses can produce synergistic cytotoxic action. This original finding is the rationale for an ongoing phase II study of this regimen in patients presenting with colorectal cancer.

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