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Original Paper

Biochemical Mechanisms of Interferon Modulation of 5-Fluorouracil Activity in Colon Cancer Cells

C.L. van der Wilt,[1] K. Smid,[1] G.W. Aherne,[2] P. Noordhuis[1] and G.J. Peters[1]

[1]Free University Hospital, Department of Medical Oncology, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands; and [2]CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, Surrey, U.K.

The antiproliferative effect of 5-fluorouracil (5-FU) in colon cancer can be enhanced by interferons (IFN-α and IFN-γ). The mechanisms by which IFNs modulate 5-FU activity are not completely elucidated. IFN-α may elevate the levels of the active 5-FU metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) in the cell, possibly leading to increased inhibition of the target enzyme thymidylate synthase (TS), which might enhance DNA damage. It has been shown that IFN-γ can prevent 5-FU induced overexpression of TS. We studied IFN modulation in three colon cancer cell lines (SW948, WiDr, human; C26-10, murine) and the sublines WiDr/F and C26-10/F, which were adapted to low folate levels. A 1.5-fold increase in 5-FU sensitivity was observed in C26-10 and C26-10/F (by murine IFN-α,β); in SW948, WiDr and WiDr/F (by human IFN-γ) and in SW948 and WiDr/F (by human IFN-α). In none of the cell lines did human IFN-α, IFN-γ or murine IFN-α,β increase FdUMP levels after exposure to 5-FU. TS activity, indirectly measured by incorporation of [6-^{3}H]-deoxyuridine into DNA, was inhibited by 5-FU, but the IFNs did not enhance inhibition. DNA damage was measured as a drug-induced decrease of double-stranded (dss) DNA compared to control cells. After 5-FU exposure, dss DNA decreased to 60–75% in WiDr, WiDr/F and SW948 cells. Human IFN-α alone caused minimal DNA damage (95% dss DNA), but increased 5-FU-induced effects to 35–50% dss DNA. IFN-γ did not cause DNA damage and did not enhance 5-FU-mediated DNA damage. Expression of TS protein, analysed by ELISA, was increased after 5-FU exposure of SW948 cells, but this increase was not affected by addition of either IFN-α or IFN-γ. It is concluded that one of the mechanisms involved in modulation of 5-FU activity is the effect of IFN-α on 5-FU-mediated DNA damage, but for IFN-γ no mechanism of action was found. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: colon cancer, 5-fluorouracil, interferons

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INTRODUCTION

PRECLINICAL AND clinical studies have shown that interferons (IFNs) can enhance the limited antitumour activity of 5-fluorouracil (5-FU) in colon cancer [1–5]. However, recent randomised, phase III trials have shown that there is no clinical benefit for this combination [6–7]. One of the reasons is the increased toxicity observed in 5-FU/IFN-α containing regimens [6–8]. Another reason may be that there is no clear biochemical basis for this combination as there is for the combination 5-FU and leucovorin. Evaluation of a described mechanism for IFN modulation of 5-FU activity in colon cancer cells might give some explanation as to why there is no benefit for this combination.

The use of IFNs in cancer therapy was originally based on their immunomodulatory effect. Interferons are known as a family of inducible proteins which can mediate growth differentiation and immunomodulation of cells. Three major classes of IFNs can be distinguished, IFN-α, IFN-β and IFN-γ. Their response is mediated through binding to cell surface receptors, whereby IFN-α and IFN-β bind to type I receptors and IFN-γ binds to type II receptors [9]. However, the biochemical mechanisms underlying the mod-

Correspondence to G.J. Peters.
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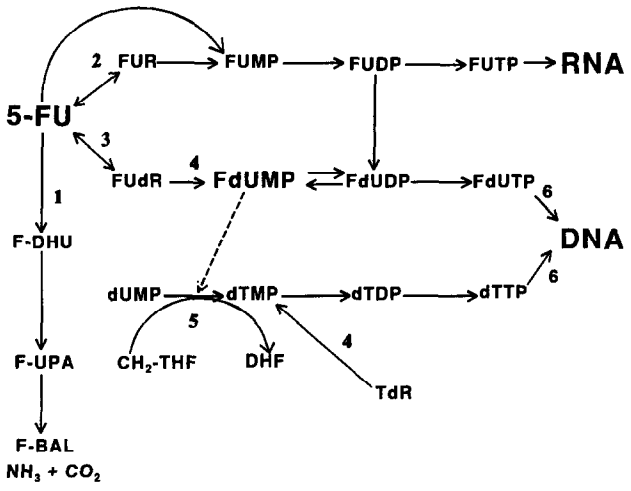

Figure 1. Anabolic and catabolic routes of 5-FU activation and degradation involved in IFN modulation of 5-FU activity. Enzymes represented by the following numbers are: 1. Dihydropyrimidine dehydrogenase; 2. Uridine phosphorylase; 3. Thymidine phosphorylase; 4. Thymidine kinase; 5. Thymidylate synthase; 6. DNA polymerase. Abbreviations: 5-FU: 5-fluorouracil; F-DHU: fluoro-dihydrouracil; F-UPA: α-fluoro-β-ureidopropionate; F-BAL: α-fluoro-β-alanine; FUR: 5-fluorouridine; FUMP: 5-fluorouridine-5′-monophosphate; FUDP: 5-fluorouridine-5′-diphosphate; FUTP: 5-fluorouridine-5′-triphosphate; FUdR: 5-fluoro-2′-deoxyuridine; FdUMP: 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUDP: 5-fluoro-2′-deoxyuridine-5′-diphosphate; FdUTP: 5-fluoro-2′-deoxyuridine-5′-triphosphate; dUMP: 2′-deoxyuridine-5′-monophosphate; dTMP: 2′-deoxythymidine-5′-monophosphate; dTDP: 2′-deoxythymidine-5′-diphosphate; dTTP: 2′-deoxythymidine-5′-triphosphate; CH_2-THF: 5,10-methylenetetrahydrofolate; DHF: dihydrofolate; TdR: thymidine.

ulating action of both interferon-α (IFN-α) and interferon-γ (IFN-γ) on the antiproliferative activity of 5-FU are not completely elucidated [5]. It seemed likely that the effect of IFN-α and IFN-β will be different from that of IFN-γ, because of the distinct receptors and also because some cells are resistant to IFN-α but not to IFN-γ modulation of 5-FU. Further understanding of the modulating mechanisms would clarify the confusion of IFN-α and IFN-γ, which occurs frequently in the literature and explain some of the disappointing results of the clinical application of the drug combination with 5-FU.

Several pathways of 5-FU anabolism have been investigated (Figure 1). The first studies on the mechanism involved in the modulating effect of IFN-α on 5-FU activity described a diminished DNA synthesis after 5-FU and IFN-α. This appeared to be related to a decreased activity of thymidylate synthase (TS). TS uses 2′-deoxyuridine-5′-monophosphate (dUMP) in the *de novo* synthesis of deoxythymidinemonophosphate (dTMP). The 5-FU metabolite, 5-fluoro-dUMP (FdUMP), acts as a fraudulent substrate and inhibits the activity of TS [10]. Decreased TS activity is apparently related to elevation of FdUMP, while dUMP levels are not affected by IFN-α [11]. A possible cause for the marked elevation of FdUMP is an IFN-α dose-dependent increase of thymidine phosphorylase and uridine phosphorylase activity [12]. Although uridine phosphorylase activity is increased, 5-fluorouridinetriphosphate (FUTP) pools are not affected by IFN-α, indicating a minor importance of this enzyme in 5-FU activation. The amount of incorporated 5-FU metabolites, FUTP and 5-fluoro-2′-deoxy-UTP (FdUTP), in RNA or DNA remain unchanged after 5-FU/IFN-α exposure [12], so it seemed unlikely that IFN-α modulation influences the RNA-directed mechanism of 5-FU mediated cytotoxicity. Also, other studies suggest that the action of IFN-α is dependent on TS inhibition mediated effects. In several cell lines, synergistic effects of 5-FU and IFN-α can be reversed by the addition of thymidine (TdR) [1, 13, 14], and no change in thymidine kinase activity has been observed [12]. However, the inhibition of TS should be obtained with a fluoropyrimidine. Growth inhibition mediated by the folate analogue N^{10}-propargyl-5,8-dideazafolic acid (CB3717) could not be modulated by IFN-α [13]. DNA damage, measured as single and double strand breaks induced by 5-FU, is increased by IFN-α modulation, and it was proposed that IFN-α enhances excision or incorporation of 5-FU bases into DNA [15]. The catabolic pathway of 5-FU has been the subject of recent mechanistic studies. The key enzyme in this pathway, dihydropyrimidine dehydrogenase, has shown decreased activity after exposure to IFN-α, resulting in increased 5-FU levels and 5-FU activity [16].

It has been shown that, in the anabolic pathway of 5-FU, IFN-γ increases the activity of uridine phosphorylase and thymidine phosphorylase [17, 18], but no changes in 5-FU nucleotides have been described. In a different study, the synergistic effects of 5-FU and IFN-γ in one of the tested colon cell lines could be explained by an overexpression of TS, which resulted from 5-FU exposure and could be reversed by IFN-γ [2, 19]. The exact regulatory role of IFN-γ at the level of TS expression has not been completely explained [19].

The new condition we introduced was the folate status of the cells. Normally, cells are cultured at supra-optimal folate concentrations compared to human plasma (2.3 μM versus 1 nM). Since the folate level might influence inhibition of TS [10], it could be important for IFN-α modulation. IFN-γ effects on TS overexpression may also be dependent on the folate level, since TS protein bound to the reduced folate cosubstrate cannot bind to TS mRNA [20]. Although the regulation of TS mRNA and protein levels have not been studied under low folate conditions, it is possible that, under these conditions, more free TS protein exists, which prevents an overexpression of TS mRNA.

This study was performed to determine whether biochemical mechanisms which are described in only one or two cell lines are generally biochemical mechanisms for IFN-α and IFN-γ modulation of 5-FU activity in various colon cancer cells. For example, it is commonly assumed that modulation by IFN-γ will prevent an increase in TS protein levels after 5-FU exposure. However, this has only been shown for one cell line so far. We tested whether this was a common phenomenon in colon cancer cells. Furthermore, IFN-γ was used in the assays for comparison with IFN-α and to see whether common mechanisms existed. Also, combinations of both IFN-α and IFN-γ were used to see whether the effect of one could be increased or abrogated by the other.

MATERIALS AND METHODS

Chemicals

5-FU was obtained from Sigma (St Louis, Missouri, U.S.A.) and stock solutions were dissolved in sterile 0.9%

NaCl at a concentration of 10^{-2} M and stored at −20°C. Human IFN-α_{2a} was from Hoffman LaRoche (Mijdrecht, The Netherlands); human IFN-γ (hIFN-γ) was a gift from Boehringer Ingelheim (Alkmaar, The Netherlands); murine IFN-α,β (mIFN-α,β) was from Sigma. Deoxy-[6-^{3}H]-uridine (^{3}H-dUrd) (25 Ci/mmol) was obtained from Amersham (Den Bosch, The Netherlands). [2-^{14}C]-thymidine (^{14}C-TdR) (59.3 mCi/mmol) was from Dupont de Nemours NEN (Dordrecht, The Netherlands). [6-^{3}H]-FdUMP was from Moravek (Brea, California, U.S.A.). All other chemicals were of analytical quality and commercially available.

Cell lines

We use three colon cancer cell lines, one of murine origin (C26-10) [21, 22] and two of human origin (WiDr and SW948) [23, 24]. These cells were routinely cultured in Dulbecco's modified Eagles medium (Gibco) with 5% v/v fetal calf serum (FCS) (Gibco). This medium contains approximately 2.3 μM folates. To adapt two of the cell lines, C26-10 and WiDr, to low folate conditions, we gradually decreased the folate content [25].

Growth inhibition test

We used the SRB test, as originally described by Skehan and associates [26], and as modified by Keepers and associates [27], to evaluate the antiproliferative effects. Briefly, cells were seeded in triplicate in 96-well flat bottom plates (Greiner, Alphen a/d Rijn, The Netherlands) in different densities depending on their growth rate (C26-10: 2000 cells/well; WiDr, C26-10/F and SW948: 5000 cells/well; WiDr/F: 10000 cells/well). After 24 h, drug-containing medium was added. 5-FU was used in concentrations ranging from 10^{-4} to 10^{-8} M. Human IFN-α_{2a} and hIFN-γ were used at a concentration of 500 U/ml and 100 U/ml, respectively. The final concentration of mIFN-α,β, used for C26-10 and C26-10/F, was 500 U/ml. 5-FU and IFNs were added simultaneously. TdR rescue was used to evaluate whether growth inhibitory effects of 5-FU were mediated by TS inhibition and, therefore, 10 μM TdR was added simultaneously with either drug alone or the combination of 5-FU and IFNs. After a drug exposure time of 72 h, cells were fixed with trichloroacetic acid (TCA) and stained with the sulforhodamine B protein dye. The results were expressed as percentage of control growth based on the difference in optical density (OD) at the start and end of drug exposure according to the formula [28]:

$$\{(\text{OD treated}/\text{OD start drug exposure}) - 1\}/$$

$$\{(\text{OD control}/\text{OD start drug exposure}) - 1\} \times 100\%$$

Incorporation of ^{3}H-UdR and ^{14}C-TdR

The assay measuring incorporation of ^{3}H-UdR into DNA has been described in detail previously [29]. Cells were seeded into 96-well plates with a filter bottom and were exposed to 5 or 10 μM 5-FU with or without 5000 U/ml hIFN-α_{2a}, 100 U/ml hIFN-γ or 500 U/ml mIFN-α,β (murine cells) for 3 h. For the last 2 h of the incubation, ^{3}H-dUrd (final concentration 0.5 μM; specific activity 6 Ci/mmol) was added. Cells were precipitated with 8% TCA on to the filter. After several washes with water, the filter was removed and transferred to a liquid scintillation vial. NaOH (2 M) was used to solubilise the nucleic acids and finally radioactivity was counted. Incorporation of ^{14}C-TdR was determined in essentially the same way. Instead of ^{3}H-dUrd, ^{14}C-TdR (final concentration 2.8 μM; specific activity 59.3 mCi/mmol) was added after a 1-h drug exposure. It was not necessary to elute the filters with precipitated cells in 2 M NaOH, because the ^{14}C label and radioactivity could be measured directly after the washes.

Free FdUMP levels

Accumulation of free FdUMP in the cells was measured according to a procedure published previously [30, 31]. After an incubation of 2 h with 10 μM 5-FU with or without 5000 U/ml hIFN-α_{2a}, 100 U/ml hIFN-γ or 500 U/ml mIFN-α,β at 37°C, cells were centrifuged and the pellet put on ice. Extraction of FdUMP was performed with 80% TCA as previously described [31] and subsequently neutralised by trioctylamine and 1,1,2-trifluorotrichloroethane. Samples were analysed in a isotope dilution assay using ^{3}H-FdUMP and *Lactobacillus casei* TS.

Fluorometric analysis of DNA unwinding (FADU) assay

Formation of DNA strand breaks was determined with the FADU assay described by Birnboim and Jevcak [32] and slightly modified by Meijer and associates [33] and Bergman and associates [34]. This assay is based on the principle that the extent of DNA unwinding in an alkali environment is related to the number of strand breaks present in the DNA. So more breaks mean that more unwinded DNA can be detected with ethidium bromide (EtBr). Cells cultured in flasks were exposed to drugs for 72 h at the following concentrations: 5-FU 1 μM (SW948) and 5 μM (WiDr and WiDr/F); hIFN-α 5000 U/ml and hIFN-γ 100 U/ml. At the end of this period, cells had grown to confluency in the flasks. Etoposide (VP16) was used as a control drug—50 μM added to cells 1 h before harvesting causes strand breaks. Untreated cells were used as controls. Each sample was divided into three parts to measure fluorescence of denatured DNA (B:blank) and not unwound DNA (T:total) in order to correct the values of the actual drug effect in the third part (P). The percentage of ds-DNA was calculated by $(P-B)/(T-B) \times 100\%$.

ELISA

Partly purified TS from a TS overproducing lymphoblastoid cell line W1L2:C1 [35] was used as a standard to evaluate TS protein levels after different drug exposures. Purified TS, standardised against pure human recombinant TS (1 fmol = 72 pg), was used to coat a 96-well ELISA plate (maxisorb NUNC-immunoplate, GIBCO, Breda, The Netherlands) in 150 μl coating buffer (70 mM sodium barbitone, 1 mM dithiotriethol (DTT)) for 2.5 h at 37°C in a humid atmosphere. The final concentration of standard TS was 0.025 ng/μl. Then the plate was washed three times with a 1% Tween 20 solution. Standard TS, in concentrations varying from 0.05 to 50 pg/μl, was added to triplicate wells to produce a standard curve. Standard TS was diluted in 100 μl of buffer containing 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 1 mM DTT, 1 g/l gelatin, 0.1 g/l thiomersal and 0.05% Tween 20. Samples were cell pellets of at least 5×10^6 cells. These were suspended in diluting buffer without gelatine and sonicated for 3×10 sec. Three cell concentrations were tested

per sample (2, 1 and 0.5×10^6 cells/100 µl) in duplicate to check linearity. Thereafter, the first antibody, a polyclonal rabbit-antihuman TS antibody [36] (50 µl, 1/10K) was added to all wells except at the non-specific (NSB) wells. The contents of the wells were mixed and incubated overnight. The next day the plate was washed three times again with Tween solution. Then the second antibody, goat-antirabbit IgG linked to peroxidase (DAKO A/S, Glostrup, Denmark) (150 µl, 1/10K) was added to the wells and incubated for 1 h. Then the plate was washed three times with 1% Tween and subsequently, 150 µl of the working solution tetramethylbenzidine (TMB) was added to the plate. The working solution of TMB consisted of 100 µl of a 83 mM TMB solution in dimethylformamide, 20 ml 25 mM citrate acid, H_2O, 50 mM Na_2HPO_4 and 0.01% (v/v) H_2O_2. The plate was incubated for 15–30 min at 37°C, until a visible blue colour appeared. Further development of the blue colour was stopped by addition of 50 µl 1 M HCl, which turned the colour yellow. Optical density was measured at 450 nm and values were zeroed on NSB wells. The B_0 value (wells with no standard TS) with the optimal density obtained from the coated TS was set at 100%. Values of standard curve (B) were calculated as $B/B_0 \times 100\%$ and plotted against log pg standard TS added. The B/B_0 values for samples were interpolated from this curve. Drug exposure of cells was comparable to growth inhibition experiments.

RESULTS

Growth inhibition

The antiproliferative effect of hIFN-α, mIFN-α,β and hIFN-γ was tested in each of the cell lines. Human IFN-α at concentrations of 500–5000 U/ml and mIFN-α,β at 500 U/ml were not growth inhibitory for the cells; 100 U/ml hIFN-γ caused minimal antiproliferative effects (90% of control growth) (data not shown). The effect of 5-FU on C26-10 and C26-10/F cells could only be enhanced slightly by mIFN-α,β and not by hIFN-α (Table 1). A control experiment with pure mIFN-α (data not shown) excluded specific effects of mIFN-β. For the various human cell lines, different modulation effects were observed. In contrast to WiDr cells WiDr/F cells were sensitive to hIFN-α modulation of 5-FU activity. Both cell lines were sensitive, but not significantly, to hIFN-γ modulation and the IC_{50} for 5-FU decreased 1.5-fold. The antitumour activity of 5-FU on SW948 cells could be modulated with both hIFN-α and hIFN-γ. A combination of both hIFNs enhanced the modulation capacity in SW948 and WiDr/F cells compared to each IFN alone. Addition of 10 µM TdR partly reversed the antiproliferative effect of 5-FU in most of the cell lines (Table 2). The modulating effect of hIFN-α and mIFN-α,β could be reversed, but modulation by hIFN-γ could only be reversed in WiDr and WiDr/F cells. The growth inhibitory effect of single high-dose hIFN-α, hIFN-γ or mIFN-α,β (not used for modulating experiments) could not be reversed by TdR.

TS activity

Growth inhibitory effects were examined at a concentration of 500 U/ml hIFN-α which is clinically achievable, but biochemical effects were studied at 5000 U/ml to be able to detect maximal effects. It has been shown that hIFN-α modulation in SW948 cells is dose-dependent [14], so we expected to see larger biochemical effects at higher dose hIFN-α.

TS activity, as measured indirectly by the incorporation of ^{3}H-dUrd into DNA, was inhibited partly by 5 µM (56%) and 10 µM 5-FU (32%) in C26-10 cells (Figure 2a). In WiDr cells, these values were 62% at 5 µM and 16% at 10 µM 5-FU, but in the low folate variants only a small difference between 5 and 10 µM was observed; C26-10/F: 51 and 50%, respectively, and WiDr/F: 47 and 35%, respectively. SW948 were more sensitive, 5 µM 5-FU caused nearly complete inhibition of ^{3}H-dUrd incorporation (17%), but 10 µM did not cause more inhibition (14%). Addition of IFNs did not enhance the effect of 5-FU on C26-10 cells (Figure 2a) and similar effects were observed for the other cell lines (data not shown). Murine IFN-α,β or hIFN-γ alone stimulated the incorporation of ^{3}H-dUrd in C26-10 cells, but not in the other cell lines (data not shown).

In a series of experiments, we measured whether a decrease of ^{3}H-dUrd was not accompanied by a decrease in thymidine kinase (TK) activity, which would mask modulating effects of IFNs. TK catalyses the conversion of dUrd into dUMP, the substrate of TS. TK also catalyses the first step of conversion of TdR into dTMP, a precursor for DNA components (Figure 1). Exposure to 5-FU increased the incorporation of ^{14}C-TdR into DNA (Figure 2b) in all the colon cells (only results for C26-10 all line shown), proving that TK activity did not decrease dramatically and no influence of the IFNs could be detected in the cell lines.

FdUMP levels

For all cell lines, we investigated whether IFNs could increase FdUMP levels, since the potentiating effect of IFN-α might be caused by an increase of FdUMP [11]. The amount of free FdUMP formed from 10 µM 5-FU varied per cell line (Table 3). Striking differences were observed between wild-type cells and low folate variants

Table 1. Effect of several IFNs on growth inhibition of 5-FU

	5-FU	5-FU + IFN-α† (500 U/ml)	5-FU + hIFN-γ (100 U/ml)	5-FU + hIFN-α + γ (500 + 100 U/ml)
Murine cell lines				
C26-10	0.5 ± 0.2	0.3 ± 0.2	0.8 ± 0.5	0.5 ± 0.3
C26-10/F	0.6 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
Human cell lines				
WiDr	7.0 ± 3.0	9.6 ± 1.0	4.5 ± 1.0	7.4 ± 2.6
WiDr/F	7.5 ± 3.5	4.9 ± 3.2	4.9 ± 3.2	1.2 ± 0.9*
SW948	1.8 ± 0.7	0.6 ± 0.3*	1.1 ± 0.4	0.4 ± 0.1*

Values are IC_{50} in µM and the mean ± S.D. of five to six experiments each performed in triplicate.
*Significantly different from 5-FU only ($P < 0.01$ Student's t-test). †In murine cells, mIFNα,β was used and in human cells, hIFN-α.

(Table 3). In WiDr/F and C26-10/F cells, higher free FdUMP levels were formed than in WiDr and C26-10. Obviously, the rate of 5-FU anabolism to FdUMP differed per cell line. The expected increase of free FdUMP after 5-FU/IFN-α was absent. In four of the five cell lines, the combination 5-FU/IFN-α produced even lower free FdUMP levels than 5-FU alone. Only in C26-10 cells was a notable increase observed for the combination IFN-α and IFN-γ. Statistical analysis by Students *t*-test for paired data of the absolute values revealed no significant differences.

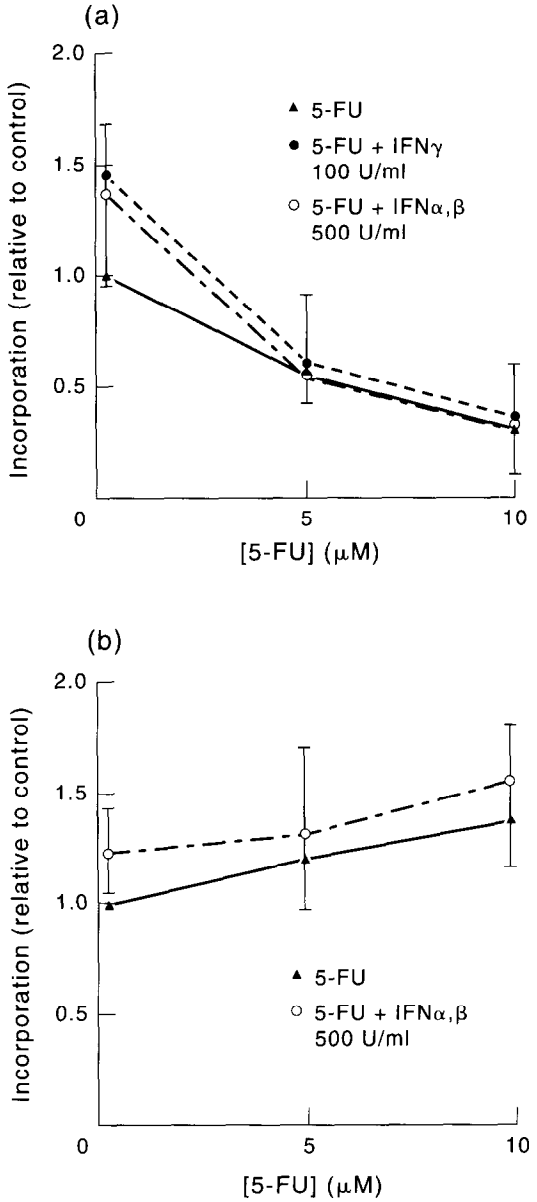

Figure 2. Incorporation of (a) ^{3}H-dUrd and (b) ^{14}C-TdR into DNA of C26-10 murine colon cancer cells, when exposed to FU (▲) or a combination of 500 U/ml mIFN-α,β (○) or 100 U/ml hIFN-γ (●) and 5-FU. Values are means ±S.D. of 3-4 experiments, expressed as a relative value of dpm incorporated in control cells (without drugs) and corrected for non-specific effects of ^{3}H-dUrd. Control values for ^{3}H-dUrd and ^{14}C-TdR incorporation were at least 0.2 and 25 000 pmol/h/10^{6} cells, respectively, for C26-10 cells.

*Table 2. TdR rescue of antiproliferative effects**

	5-FU	5-FU + IFN-α†	5-FU + hIFN-γ
Murine cell lines			
C26-10	1.5 ± 0.3	1.5 ± 0.3	1.2 ± 0.3
C26-10/F	1.3 ± 0.3	1.2 ± 0.1	1.1 ± 0.1
Human cell lines			
WiDr	1.0 ± 0.4	1.7 ± 0.4	1.7 ± 0.2
WiDr/F	1.4 ± 0.3	1.6 ± 0.4	1.5 ± 0.3
SW948	1.9 ± 0.3	1.5 ± 0.1	1.0 ± 0.2

*Values are expressed as a modifying factor calculated from the relative growth of cells treated with 5-FU + 10 μM TdR/relative growth of cells treated with 5-FU alone. This was calculated at the approximate IC_{50} concentration of 5-FU for each cell line (C26-10 and C26-10/F: 0.5 μM; for WiDr and WiDr/F: 5 μM; for SW948: 2.5 μM). †In murine cells, mIFNα,β was used and in human cells, hIFN-α. IFN concentrations were: mIFN-α,β and hIFN-α: 500 U/ml; hIFN-γ: 100 U/ml. Values are the mean ± S.E. of three to four paired experiments performed in triplicate

DNA strand breaks

Since the potentiating effect of IFN may be caused by increased DNA damage, drug-induced DNA damage was evaluated. The percentage of dss DNA in drug-treated cells was compared to that in control cells. 5-FU exposure for 72 h caused a decrease in dss DNA to 62%, 80% and 77% in WiDr, WiDr/F and SW948 cells, respectively (Figure 3). Neither hIFN-α nor hIFN-γ caused significant DNA damage in these cell lines. The combination 5-FU/hIFN-α was very active and decreased dss DNA to 52%, 35% and 49% in SW948, WiDr and WiDr/F cells, respectively. Also, in the combination 5-FU/hIFN-α and γ, modulation of the 5-FU activity was detected, but 5-FU/hIFN-γ was not better than 5-FU alone in all cell lines. Differences between effects of 5-FU and 5-FU/hIFN-α were statistically significant, $P < 0.05$ (Students *t*-test for paired data).

TS protein expression

In SW948 cells, we evaluated the protein expression of TS after 72 h exposure to 5-FU (Figure 4). These cells were sensitive to modulation by IFN-γ, which has been reported to prevent increase of TS protein after 5-FU exposure [20]. We observed a slightly enhanced expression of TS protein after exposure to 5-FU or 5-FU–IFN combinations compared with TS expression of control cells, but the difference was not significant. After exposure to 5-FU and hIFN-α or hIFN-γ, TS protein levels were somewhat higher than after 5-FU alone, so the expected prevention was clearly not present. The IFNs alone did not affect TS protein expression in this cell line.

DISCUSSION

The biochemical mechanisms of IFN-α or IFN-γ modulation of 5-FU activity were studied in five different colon cell lines. Numerous *in vitro* studies report synergistic effects of the combination 5-FU and IFN-α, but recent clinical trials in colorectal cancer show no advantage for the combination 5-FU and IFN-α [6, 7]. Although the clinical relevance of the combination has become minimal, it could be useful to examine why it did not reach the promising results of early studies. For instance, dose and scheduling based on a sound biochemical rationale for the combination are mandatory to translate the *in vitro* results to clinical data.

Table 3. Effect of exposure to IFNs on free FdUMP levels in colon cancer cell lines

	Free FdUMP		
	5-FU + IFN-α	5-FU + hIFN-γ	5-FU + hIFN-α + γ
Murine cell lines			
C26-10	0.52 ± 0.07 (3)	1.15 ± 0.29 (4)	1.55 ± 0.32 (4)
C26-10/F	0.80 ± 0.16 (4)	0.82 ± 0.09 (5)	0.72 ± 0.13 (5)
Human cell lines			
WiDr	1.21 ± 0.30 (6)	0.69 ± 0.1 (4)	1.18 ± 0.08 (5)
WiDr/F	0.92 ± 0.11 (4)	1.06 ± 0.08 (6)	0.92 ± 0.07 (5)
SW948	0.76 ± 0.15 (4)	1.16 ± 0.09 (5)	1.17 ± 0.23 (4)

Values are means ± S.D. of (*n*) experiments. FdUMP levels were expressed relative to values measured after 2 h exposure to 10 μM 5-FU alone. Absolute values of FdUMP levels were: 72 ± 16 pmol/10^6 cells (6) (C26-10); 440 ± 43 pmol/10^6 cells (5) (C26-10/F); 147 ± 9 pmol/10^6 cells (6) (WiDr); 1401 ± 67 pmol/10^6 cells (6) (WiDr/F) and 73 ± 10 pmol/10^6 cells (6) (SW948). IFN concentrations were: 500 U/ml mIFN-α,β for murine cells, 5000 U/l hIFN-α for human cells, 100 U/ml hIFN-γ for all cells.

Several mechanisms have been proposed, but we found only one that is common for several colon cell lines: drug-induced enhancement of DNA damage. However, this mechanism only partly explained the growth inhibitory effects of 5-FU and IFN-α and not the effects of 5-FU and IFN-γ.

In general, the modulating effect of IFNs on 5-FU-induced growth inhibition was small, approximately 1.5-fold, but this was comparable to values reported elsewhere [14, 37]. Modulation of 5-FU activity in the murine cell lines was only possible with murine IFN-α,β, which suggests a species-specific effect. These observations are in line with a study of Elias and Crissman [1], who described better effects for murine than human IFN-α in modulation of 5-FU activity in murine MCA-38 cells. IFN-γ is known to be species-specific as an immunomodulator [38] and the lack of activity of human IFN-γ in murine C26-10 cells was not surprising. Adapting WiDr cells to low, more physiologic folate levels increased their susceptibility to IFN-α modulation of 5-FU-mediated growth inhibition. The sensitivity of 5-FU itself was not changed in WiDr/F compared with WiDr cells [39]. WiDr/F cells grew slower than WiDr cells, but had a much higher activity of TS (unpublished data). Thus, it seems unlikely that the level of TS activity determined the sensitivity to hIFN-α modulation. The SW948 cell line, which was sensitive to both hIFN-α and hIFN-γ modulation, has been studied for dose-dependent hIFN-α modulation [14], and a 2-fold difference was observed between 5-FU IC_{50} of combinations with 500 and

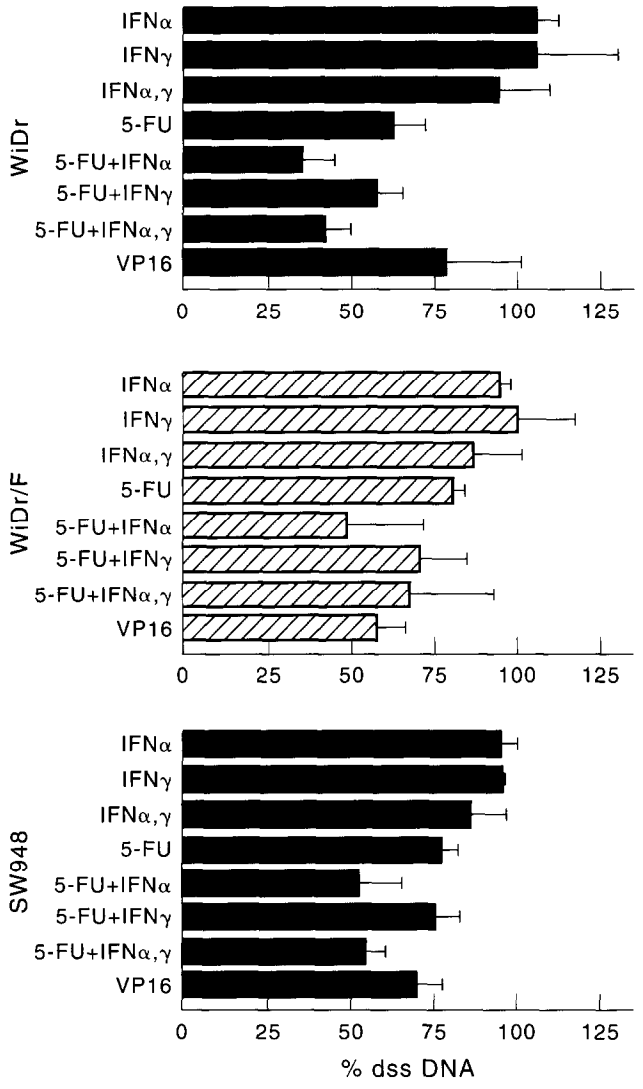

Figure 3. Drug-induced DNA damage in WiDr, WiDr/F and SW948 cells after 72 h drug exposure. Concentrations of drugs were FU 1 μM (SW948) or 5 μm (WiDr, WiDr/F), hIFN-α 5000 U/ml and hIFN-γ 100 U/ml, VP16 50 μM (1 h). Values are means ± SD of four experiments.

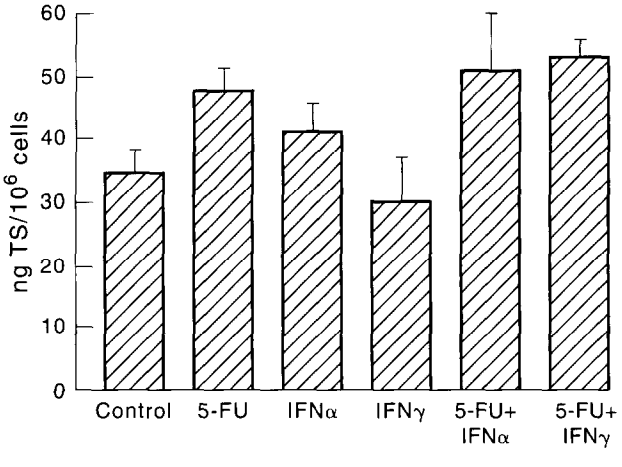

Figure 4. Expression of TS protein after drug exposure of SW948 colon cancer cells. Values are means ± SD of three series of experiments. Control value was 34.7 ± 3.7 ng TS/10^6 cells or 99 ± 7.7 ng TS/mg protein. TS levels after FU + hIFN-γ were significantly higher than control ($p < 0.05$ Student's *t*-test for paired data).

5000 U/ml hIFN-α. This was consistent with data from Houghton and associates [13]. hIFN-α modulation of 5-FU-induced growth inhibition could be rescued with 10 μM TdR in the human cell lines, indicating that TS might play a role in hIFN-α modulation. The antiproliferative effect of a single dose of 5-FU in the WiDr cells seemed to be directed towards the RNA, because minimal rescue of TdR was observed. Modulation by hIFN-α and hIFN-γ directed it more towards TS as can be deduced from measurable TdR rescue.

Indirect measurements of TS inhibition by ^{3}H-dUrd incorporation revealed no changes in the extent of TS inhibition after IFN modulation in either of the cell lines. An increased TS inhibition after IFN-α/5-FU in HL60 cells had been reported by Elias and Sandoval [11]. The increased ^{3}H-dUrd incorporation in C26-10 cells after single mIFN-α,β or hIFN-γ is in contrast to other observations. A reduction of [^{3}H]uridine and [^{3}H]TdR incorporation into DNA of Daudi cells after human lymphoblastoid IFN exposure was described by Gewert and associates [40]. We observed no specific effects of IFNs on ^{14}C-TdR incorporation in our colon cell lines in contrast to the results of Gewert and associates [40]. Our data corresponded well to those of Schwartz and associates [12], describing no change in TK activity after IFN-α modulation of 5-FU in HT29 colon cancer cells and Elias and Sandoval [11] describing similar effects on HL60 cells. A study performed with TK-deficient cells suggest that changes in TdR utilisation are not involved in the IFN-α modulation of 5-FU [13].

A direct effect of hIFN-α or hIFN-γ modulation on TS inhibition could not be related to an elevation of free FdUMP concentrations. Cells that were sensitive to IFN-α modulation showed no increase in free FdUMP, unlike HT29 colon cancer cells [12]. Stimulation of the metabolic activation of 5-FU seemed to play a minor role in the enhanced growth inhibition after 5-FU exposure in combination with IFNs. It did not lead to significant changes in TS activity [41]. In contrast, the increase of thymidine phosphorylase and uridine phosphorylase activity after modulation with hIFN-α or hIFN-γ had a greater impact on the susceptibility of colon cells for the prodrug of 5-FU, 5'-deoxy-5-fluorouridine, than that of 5-FU itself [18, 42]. This is one of the few effects on 5-FU metabolism that has been reported for both hIFN-α and hIFN-γ.

The most well-known explanation for the modulating effect of hIFN-γ has been described by Chu and associates [2]. They showed that exposure to 5-FU resulted in a 3-fold increase of TS protein in H630 colon cancer cells, that could be prevented by hIFN-γ. High TS protein levels, associated with a higher enzyme activity, resulted in a high IC_{50} for 5-FU in several cell lines [43]. Although TS expression seemed to depend on time after transfer and 5-FU exposure [44], we found that TS protein levels were only moderately elevated by 5-FU exposure and no prevention by hIFN-α or hIFN-γ was observed. Therefore, we conclude that direct effects on TS protein or activity could be excluded as general mechanism of hIFN-α or hIFN-γ modulation, as already proposed by Houghton and associates [13].

The main mechanism of action that can explain the activity of the combination 5-FU–hIFN-α was DNA damage. 5-FU-induced DNA damage can be caused by a depletion of dTMP resulting in short-chain DNA or increased misincorporation of dUTP and FdUTP resulting in a defective strand, which cannot be copied or become double stranded. Modulation by IFN-α may affect the repair of these DNA strand breaks. It has been reported that DNA polymerase-α and -β activity can be inhibited by hIFN-α [45]. Increased DNA double strand breaks after 5-FU–hIFN-α than after 5-FU alone was also described for three other colon cancer cells by two different research groups [15, 46], using different methods. It remains unclear why modulation by hIFN-α increased DNA damage in WiDr cells, but did not enhance growth inhibition. Probably repair of the induced DNA damage is also important. In WiDr/F cells, DNA damage was also increased after hIFN-α, but this did result in enhanced growth inhibition. A possible explanation could be availability of thymidine nucleotides, which will be limiting in the low folate adapted cells. Folate status is not the only reason, because in SW948 cells a similar reaction was found. Further research on, for example, DNA polymerase or uracil DNA glycosylase activity in WiDr and SW948 might explain the difference.

The general mechanism of IFN-α modulation of 5-FU activity seems to be mediated by increased DNA damage, but the process by which these strand breaks are formed and cause growth inhibition is unclear and has to be further investigated. However, mechanisms by which hIFN-γ modulates 5-FU activity seem to be very different from that of hIFN-α. No common biochemical effect could be shown. Besides the one, very elegant mechanism by which hIFN-γ modulation of 5-FU has been explained for one cell line [2], no other biochemical mechanism has been detected which is applicable to our colon cancer cells. These results showed a hiatus between INF modulatory activity and biochemical mechanism: modulatory activity (IFN-γ) but no mechanism, or a mechanism (WiDr, IFN-α) but no modulatory activity. One of the implications of the latter could be that such variability only would increase toxicity and decrease therapeutic efficacy of IFN-α modulation of 5-FU activity in the treatment of advanced colorectal cancer.

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