Pergamon

European Journal of Cancer Vol. 31A, Nos. 7/8, pp. 1316–1320, 1995
Elsevier Science Ltd
Printed in Great Britain

0959-8049(95)000175-1

The Role of Interferon-α as a Modulator of Fluorouracil and Leucovorin

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Several preclinical studies have demonstrated that interferon-α (IFN-α) may enhance the cytotoxicity of fluoropyrimidines in a greater-than-additive manner in a variety of human cancer cell lines. The underlying mechanism(s) have varied in different cancer cell lines, and include increased fluorouracil anabolism to fluorodeoxyuridine monophosphate, further inhibition of thymidylate synthase, stimulation of thymidine and uridine phosphorylase activities, greater DNA damage, and enhanced natural killer cell-mediated lysis of tumour targets. These preclinical studies stimulated clinical evaluation of IFN-α in combination with 5-fluorouracil (5-FU) with and without leucovorin (LV), and the initial clinical results appeared promising. We summarise preclinical research concerning the interaction of 5-FU and IFN-α. The rationale for combining 5-FU with IFN-α and LV is discussed, and we describe our clinical experience with the combination of 5-FU, LV and IFN-α-2a. The insights and unresolved questions concerning the clinical application of this combination are also discussed.
Eur J Cancer, Vol. 31A, Nos 7/8, pp. 1316–1320, 1995

PRECLINICAL STUDIES OF 5-FLUOROURACIL (5-FU) AND INTERFERON (IFN)-α

INTERFERONS (IFN) are a family of proteins that regulate a wide spectrum of cell functions and modulate host responses to infection and malignancy. The binding of type I (α and β) and type II (γ) IFN to high-affinity cell surface receptors activates a postreceptor signalling mechanism which leads to changes in gene transcription, protein expression, enzyme activities, nucleotide pools and cell cycle distribution. The *in vivo* immunomodulatory consequences include effects on natural killer (NK) cells, T-cells, macrophages and induction of other cytokines. Numerous *in vitro* studies have demonstrated that each type of IFN may interact with fluoropyrimidines in a greater-than-additive manner to produce cytotoxicity in a variety of human cancer cell lines [1–14]. The type of IFN that maximally enhances fluoropyrimidine cytotoxicity differs among cell lines. The apparent basis for the potentiation of fluoropyrimidine cytotoxicity has also varied depending on the type of IFN used, the specific cell lines studied and the duration of drug exposure.

Elias and Crissman reported that IFN-α (100 U/ml) augmented 5-FU cytotoxicity in HL–60 promyelocytic leukaemia cells. The degree of potentiation increased with increasing duration of exposure and maximal effects (a 3.2-fold enhancement of 5-FU toxicity) were seen with a 6–7 day exposure to both drugs [1]. The underlying mechanism appeared to be an IFN-α-mediated increased formation of the active metabolite, fluorodeoxyuridine monophosphate (FdUMP), associated with increased inhibition of thymidylate synthase (TS) [2]. Schwartz and associates also found that concurrent exposure to IFN-α and 5-FU led to a 2-fold increase in FdUMP levels in a colon cancer cell line [4]. IFN-α and -γ exposure may be accompanied by an increase in the activities of uridine and thymidine phosphorylase, an effect that may contribute to increased 5-FU anabolism [5, 6].

The ability of thymidine to rescue cells from the additive effects of IFN has led to the conclusion that IFN enhances the DNA-directed actions of 5-FU and fluorodeoxyuridine [1, 7, 9, 10]. Chu reported that resistance of NCI–H630 colon cancer cells to 5-FU was accounted for by 3–5.5-fold increase in TS content during 5-FU exposure, accompanied by partial recovery of functional TS [7]. IFN-γ abrogated the increase in TS content induced by 5-FU, thus resulting in enhanced inhibition of TS and augmentation of 5-FU cytotoxicity [7, 8]. In GC3/cl human colon cancer cells, in contrast, the locus of interaction between IFN-α and 5-FU appeared to be at the level of parental DNA damage in the absence of an effect on 5-FU metabolism or the extent of TS inhibition [9, 10]. Both DNA single-strand and double-strand breaks were significantly enhanced by IFN-α, and the addition of LV to 5-FU/IFN-α further potentiated DNA damage [10].

We found that pre-exposure of HT-29 colon cancer cells to 500 U/ml IFN-α-2a for 24 h followed by concurrent exposure to 5-FU and IFN-α-2a for an additional 72 h enhanced the cytotoxicity of 5-FU [11]. IFN-α did not increase either 5-FU ribonucleotide metabolism or 5-FU-RNA incorporation [11]. Total TS protein content increased approximately 1.4-fold after a 24 h exposure to 1 μM 5-FU. A 48 h exposure to 500 U/ml IFN-α-2a did not appreciably affect TS content when given alone, and did not abrogate the 5-FU-associated increase in TS content. TS catalytic activity in cell lysates was decreased to 29%

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of control after a 24 h exposure to 1 μM 5-FU, but the addition of IFN-α-2a 500 U/ml (0–48 h) did not potentiate TS inhibition (25% of control) (Grem *et al*, National Cancer Institute, U.S.A.).

We studied the effects of 5-FU ± IFN-α on newly synthesised DNA replication intermediates using pH step alkaline elution. With this technique, interference with DNA chain elongation (through substrate depletion or damage to nascent DNA) is characterised by an increased proportion of radiolabelled DNA eluting with sequential 1 h exposure to buffers with progressively more alkaline pH [12]. In control cells, 45% of the total nascent DNA was retained on the filter. IFN-α-2a by itself (500 U/ml 0–48 h) produced minimal effects compared to control (40% nascent DNA retained, 89% of control) [11]. With 1 μM 5-FU (24–48 h), increased elution of nascent DNA was evident (29% nascent DNA retained, 64% of control); more pronounced effects were seen with the combination of 5-FU plus IFN-α-2a (17% nascent DNA retained, 38% of control) (Grem *et al*, National Cancer Institute, U.S.A.). Thus, greater interference with nascent DNA replication intermediates may contribute to the enhanced cytotoxicity seen with the combination. Additional studies are in progress to assess damage to parental DNA.

The preclinical data, therefore, suggest that IFN-α may enhance the DNA-directed toxicities of 5-FU. By increasing the intracellular content of reduced folates, leucovorin (LV) enhances the formation and stability of the ternary complex between thymidylate synthase, FdUMP and the reduced folate cofactor 5, 10-methylenetetrahydrofolate. The combined use of LV and IFN-α, therefore, represents a reasonable approach to modulating the DNA-directed cytotoxic effects of 5-FU. Indeed, Houghton and associates have reported that in the presence of both IFN-α and 1 μM LV, much greater potentiation of 5-FU cytoxicity was seen compared to that achieved with 5-FU and IFN-α alone [9]. A concurrent 72 h exposure to 5-FU with IFN-α 500 and 500 U/ml enhanced 5-FU toxicity by 3.2- and 3.4-fold in a colony formation assay, respectively. The addition of 1 μM LV to either 50, 500 or 5000 U/ml IFN-α enhanced 5-FU cytotoxicity by 4.6-, 10.9- and 13.8-fold, respectively [9]. These investigators have confirmed that the combination of 5-FU/LV/IFN-α resulted in superior antitumour activity in colon cancer xenografts compared with the use of either as a single modulator of 5-FU [13].

An alternate hypothesis for the interaction has been suggested in other studies. Neefe and colleagues reported that overnight incubation of natural killer (NK) cells with IFN-α resulted in enhancement of NK cell-mediated cytoxicity of melanoma tumour target cells [14]. However, incubation of the target cells with IFN-α paradoxically induced resistance of the cancer cells to NK cell-mediated lysis. In contrast, incubation of the tumour target cells with 5-FU abrogated the IFN-induced resistance to IFN-activated NK cell lysis [14].

Various IFNs or IFN inducers have been reported to improve the therapeutic index of 5-FU in tumour-bearing mice by either increasing the antitumour activity or reducing host toxicity; in the latter case, induction of a proliferative arrest in IFN-sensitive host tissues has been proposed [15]. Data from a clinical study by Cascinu and associates supports this cytokinetic hypothesis [16]. In 21 patients with operable tumours, IFN-α-2b 3 million U s.c. was given every other day in the week prior to surgery. Biopsies were obtained at endoscopy for baseline evaluation and again at surgery; the samples were taken between 9 and 11 a.m. to avoid possible circadian alterations in cell kinetics. No difference in [^{3}H]thymidine labelling index was observed in the paired tumour biopsies from 22 control patients, while the biopsies obtained post-IFN-α had a significantly decreased thymidine labelling index compared to the baseline samples (3.8% versus 13.8%, respectively) [16]. Flow cytometry studies indicated no appreciable change in the cell cycle distribution in the paired samples from control patients. In contrast, the post-IFN-α tumour biopsies showed an accumulation of cells in G_0–G_1 with decreased proportion in S phase (baseline, post-IFN): G_0–G_1, 70% versus 79%; S phase, 25% versus 17% [16]. These data raise the concern that continuous IFN-α administration might actually interfere with 5-FU activity by decreasing the proportion of tumour cells in the more sensitive S phase.

In summary, the underlying basis for the apparent enhancement of 5-FU cytotoxicity by IFN in preclinical studies includes several mechanisms, may differ with the type of IFN, and appears to be highly dependent on the specific cancer cell or tumour model studied. In addition to biochemical and molecular mechanisms, immunomodulatory and pharmacological effects may be operative *in vivo*.

CLINICAL STUDIES OF 5-FU, LEUCOVORIN AND IFN-α

Preclinical information suggested that the combination of 5-FU with LV and IFN-α might potentiate 5-FU activity through complementary mechanisms. Further, extended concurrent exposure to 5-FU and IFN-α (± IFN-α pre-exposure) appeared to be optimal. We, therefore, developed a regimen combining IFN-α-2a with a daily for 5 days schedule of LV (500 mg/m^2 i.v. over 30 min) and 5-FU (370 mg/m^2/day i.v. push 1 h after LV) [17]. In order to assess the impact of IFN-α-2a on the toxicity of 5-FU/LV and any potential effects on 5-FU pharmacokinetics, patients received 5-FU/LV daily for 5 days during the initial cycle. If tolerated, the same doses of 5-FU/LV were given in the second cycle, with the addition of IFN-α-2a s.c. at either 3, 5 or 10 million U/m^2/day starting 24 h prior to the first dose of 5-FU and then continued daily for 7 or 14 days. In 26 matched cycles, IFN-α-2a administration was associated with an increased incidence of dose-limiting mucositis (31%) and diarrhoea (23%) at a dose that had been tolerated the previous cycle [17]. The overall response rate was 45% in 22 previously untreated patients with advanced gastrointestinal adenocarcinoma. Responses were seen at all IFN-α-2a dose levels: with 3, 5 and 10 million U/m^2, 4 of 9, 4 of 7, and 2 of 6 patients responded, respectively. The 10 million U/m^2 dose of IFN-α-2a was clearly too toxic, while the incidence and severity of mucositis and diarrhoea appeared to be similar with 3 and 5 million U/m^2. Continuing the IFN-α-2a for 14 days offered no apparent advantage over the 7 day schedule. Since 5 million U/m^2 given on days 1–7 was associated with a biological effect relevant to 5-FU (increased 5-FU systemic exposure, discussed below), and produced similar toxicity compared to the lower IFN-α-2a dose, it was selected for further evaluation.

We further defined the activity of this regimen in a Phase II study in 46 patients with advanced colorectal cancer with measurable disease and good performance status (ECOG 0–2) [18]. Cycles were repeated at 3 week intervals if toxicity had resolved. The 5-FU dose was increased by 15% if toxicity was mild, and decreased by 15% for grade 3–4 non-haematological or grade 4 haematological toxicity. Four complete (CR) and 20 partial responses (PR) were seen among 44 assessable patients (54%) [18]. With a median follow-up of 29 months, the median time to treatment failure was 7.8 months, and median survival

was 16.3 months. Patients with clinical evidence of metastases confined to the liver appeared to have better median survival (20.1 months). Grade 3–4 non-haematological toxicity of the following types was observed (worst grade of toxicity experienced by each patient): mucositis, 37%; diarrhoea, 40%; rash, 7%; and fatigue, 14%. Serious myelosuppression was uncommon. Dose-limiting toxicity eventually required 5-FU dose reduction in 61% of patients, and 26% required a ≥25% decrease in the IFN-α-2a dose for constitutional toxicity.

We found an association between response rate and performance status. Table 1 includes all previously untreated colorectal cancer patients entered on either the pilot or Phase II study [17, 18]. Asymptomatic patients had the highest response rate (64.5%), while patients with ECOG performance status of 2 had the lowest response rate (21.4%). On the basis of this information, we currently recommend this regimen only for asymptomatic or minimally symptomatic patients.

We examined the relationship between TS expression and clinical response to therapy in surgical candidates with locally advanced adenocarcinoma of the stomach or gastro-oesophageal junction [19]. Three cycles of pre-operative 5-FU/LV/IFN-α-2a were administered. Tumour biopsies were obtained at endoscopy prior to starting therapy, and repeated during days 3–5 of the second cycle of therapy. The latter samples were obtained prior to the daily chemotherapy, approximately 20–24 h after treatment the previous day. Reduction in tumour size by ≥50% constituted a partial response (PR), while a 25–49% reduction was considered a minor response. Paired tumour biopsies were available in 13 patients (Table 2). TS content was determined by Western immunoblot analysis using the TS106 monoclonal antibody; this antibody can distinguish unbound (free) TS from bound (complexed) TS on the basis of differential migration in the polyacrylamide gel [20, 21]. Baseline TS content was 7.9-fold higher in the 5 non-responding patients compared with those who responded to neoadjuvant therapy [19]. In the tumour samples obtained during cycle 2 of therapy, total content was higher than that measured in the paired baseline samples in both groups; the absolute TS content, however, was on average 3.3-fold higher in the non-responding patients [19]. A striking difference was noted in the amount of free (unbound) TS: in non-responding patients, the absolute level of free TS was 11.3-fold higher than that in responders [19].

Table 1. Best clinical response according to performance status in patients with advanced colorectal cancer

PS	Patients	CR (%)	PR (%)	SD (%)	PD (%)
0	31	4 (12.9)	16 (51.6)	11 (35.5)	0
1	17	1 (5.9)	8 (47.1)	4 (23.5)	4 (23.5)
2	14	0	3 (21.4)	4 (28.6)	7 (50)
Total	62	5 (8.1)	27 (43.6)	19 (30.6)	11 (17.7)

The data represent patients with colorectal adenocarcinoma with no prior chemotherapy participating in two consecutive studies of 5-fluorouracil/leucovorin/interferon-α-2a [17, 18]. Stable disease (SD) was defined as too small a change to meet the requirements for partial response (PR) or progression, and the appearance of no new lesions for a period of at least 12 weeks. Early progressive disease (PD) was defined as a 25% increase over baseline tumour measurements or the appearance of new lesions within the first 12 weeks of therapy. CR, complete response; PS, performance status.

Table 2. Correlation between clinical response and thymidylate synthase (TS) protein expression

5-fluorouracil exposure	TS parameter	TS protein content (arbitrary units) No response (n = 5)	MR or PR (n = 8)	Ratio non-responders to responders
Pre	Total TS	5.5	0.70	7.9
Post	Total TS	10.2	3.1	3.3
Post	Free TS	3.4	0.3	11.3

Patients with locally advanced adenocarcinoma arising in the stomach or gastro-oesophageal junction received three cycles of 5-fluorouracil/leucovorin/interferon-α-2a at 3-week intervals prior to definitive surgery. Paired tumour biopsies were obtained from 13 patients at endoscopy prior to initial treatment, and repeated during surveillance endoscopy on days 3 to 5 of cycle two (prior to the daily dose of chemotherapy). The tumours were immediately frozen and stored at −70°C until processed. TS protein content (both bound and free) were determined by Western immunoblot analysis. Adapted from [19].

CLINICAL PHARMACOLOGY STUDIES

In conjunction with our pilot study, pharmacokinetic data were obtained in 18 matched cycles (36 cycles total) in which the same doses of 5-FU/leucovorin (LV) were administered, but IFN-α-2a was added in the subsequent cycle [17]. To allow time for any possible effect of IFN-α-2a to become evident, pharmacokinetic samples were obtained on day 4 or 5. Because of the known circadian variation in 5-FU pharmacokinetics, it was administered at the same time of day (within 2 h) for matched cycles. With each patient serving as his/her own control, a dose-dependent decrease in 5-FU clearance was noted: an increase in the 5-FU area under the plasma concentration × time curve (AUC) by 1.3- and 1.5-fold was observed in 12 patients receiving 5 or 10 million U/m²/day, whereas no such effect was apparent in 6 patients receiving 3 million U/m²/day. Six of the 36 patient cycles were complicated by grade 3 or 4 mucositis ± diarrhoea. Six of 21 cycles (29%) with an AUC ≥ 4000 μM h and three of seven cycles (43%) with an AUC ≥ 5000 μM h were associated with ≥ grade 3 toxicity while severe toxicity was not observed in 15 cycles with a 5-FU AUC < 4000 μM h. Thus, the overall enhancement of the toxicity associated with the addition of IFN-α-2a to 5-FU/LV may be explained in part by the effects of IFN-α-2a on 5-FU pharmacokinetics.

In an effort to explain the apparent effect of IFN-α-2a on 5-FU clearance, we examined the effects of IFN-α-2a on the catabolism of [³H]5-FU in intact peripheral blood mononuclear cells (PBMC) obtained from patients receiving the IFN-α-2a/5-FU/LV regimen [22]. Dihydropyrimidine dehydrogenase (DPD) catalyses the conversion of 5-FU to the "inactive" catabolite dihydrofluorouracil, and DPD activity in PBMCs appears to reflect total body enzyme activity. Although it was possible that IFN-α-2a might directly change DPD content, indirect effects brought about by perturbations in the intracellular mileau might also be possible. For example, decreased availability of NADPH cofactor or increased levels of competing substrates might influence DPD activity. Therefore, we elected to use intact PBMCs rather than cellular lysates. Matched samples from an individual patient were obtained at the same time on days 1, 2 and 4 prior to the daily doses of chemotherapy. In 47 matched patient cycles, [³H]5-FU catabolism in PBMCs was significantly decreased compared to baseline by 20% and

41% on days 2 and 4 of therapy (Figure 1) [22]. These observations suggest that changes in 5-FU catabolism during therapy with IFN-α, 5-FU and LV may account for the decreased clearance.

DISCUSSION

Preclinical information demonstrating potentiation of 5-FU cytotoxicity by IFN-α prompted clinical evaluation of the combination. The dose and schedule of IFN-α, 5-FU and LV have varied among trials, and the optimal regimen is not clear. In 1989, Wadler and associates reported that 13 of 17 untreated patients with colorectal cancer responded to a schedule of thrice weekly IFN-α-2a (9 million U/dose subcutaneously) in combination with infusional 5-FU 750 mg/m^2 for 5 days followed by a weekly bolus of 5-FU 750 mg/m^2 [23]. This exciting report led to several confirmatory phase II trials, followed by randomised clinical trials comparing 5-FU/IFN-α with either 5-FU alone (bolus and infusional regimens) or other 5-FU-modulated regimens in patients with advanced colorectal cancer. The final reports from these trials are not yet available, but preliminary results suggest no major improvement in survival with 5-FU/IFN-α compared to 5-FU alone or 5-FU/LV.

We felt there were compelling reasons to evaluate IFN-α-2a with 5-FU/LV. While our initial results showed a promising response rate, the regimen produces substantial toxicity, primarily in the form of mucositis and diarrhoea [17, 18]. Oral cryotherapy has partially ameliorated the oral mucositis, but toxicity to the epithelial lining of other gastrointestinal organs remains a problem. Although data from murine models suggested a protective effect of IFN-α [15], the toxicity of 5-FU has been greater in clinical trials with the addition of IFN-α. In general, very high doses of IFN-α (≥ 10 million U/m^2/day) have been poorly tolerated, but even lower doses (3–5 million U/m^2) are associated with increased 5-FU toxicity.

A pharmacological interaction between IFN-α and 5-FU in the clinical setting may be operative depending on the dose and/or schedule of both IFN and 5-FU and the inclusion of LV. Several investigators have reported a decrease in 5-FU clearance by IFN-α within individual patients, resulting in an increase in the 5-FU AUC, particularly with schedules that involve consecutive daily administration of IFN-α [17, 24–27]. Not all studies have identified a pharmacokinetic interaction [28, 29]. Because of considerable interpatient variability, the possible effect of a modulator on 5-FU elimination should be studied with each patient serving as his/her own control. To avoid the confounding effect of circadian variation, blood samples in matched cycles for individual patients should be obtained at the same time of day. In our pilot study, we obtained 5-FU blood samples on day 4 or 5, after the patient had received three to four consecutive daily doses of IFN-α-2a [17]. We also noted a time-dependent effect in the ability of intact PBMCs to catabolise [^{3}H]5-FU: although catabolism was decreased on day 2, the effect was even more pronounced by day 4 [22]. No such effect was observed in patients receiving 5-FU/LV alone [22]. Using a 5-day infusion of 5-FU (750 mg/m^2/day) with IFN-α-2b subcutaneously 0.1–15 million U/m^2/day, Danhauser and colleagues reported that the mean 5-FU steady-state plasma levels (*Cpss*) within the same patient before and after IFN-α were 30% higher after IFN-α administration [24]. In contrast to our findings, no apparent relationship was noted between IFN-α dose and change in 5-FU plasma levels, and a decrease in 5-FU clearance was seen with doses as low as 0.1 million U/m^2 [24]. The pharmacokinetics of 5-FU 700 mg/m^2 given as an intravenous bolus weekly in the absence of LV were studied in 12 patients who received either no IFN-α-2b, or escalating doses of 1, 5 and 9 million U total, administered three times weekly in successive cycles; the 5-FU AUC increased by 39, 45 and 92%, respectively [25, 26]. In the presence of LV, however, higher doses of IFN-α-2b were required to influence 5-FU levels. 5-FU blood samples were obtained on days 1, 3 and 5 in 8 patients receiving infusional 5-FU 750 mg/m^2/day for 5 days with IFN-α-2b 5 milllion U starting at 8 p.m. day 1, then continued daily until day 5 [27]. The 5-FU *Cpss* on day 1, prior to initiation of IFN-α-2a, was 391 ng/ml; the *Cpss* increased by 1.48- and 2.43-fold (578 and 952 ng/ml) on days 2 and 5, respectively [27]. These findings suggest that the effects of IFN-α become more pronounced over time. An unresolved question is whether such a pharmacological interaction is desirable. The correlation noted between increased

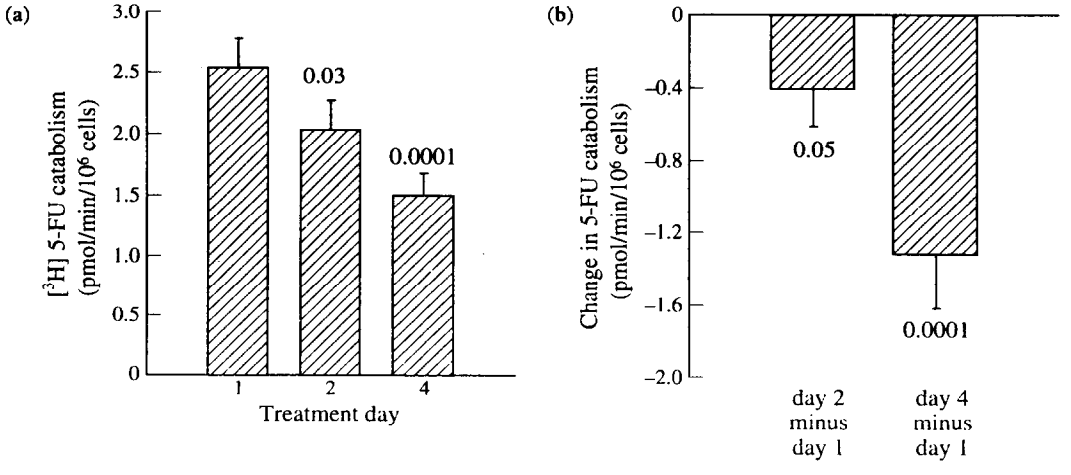

Figure 1. 5-FU catabolism in intact peripheral blood mononuclear cells during combination therapy with 5-FU, LV, and IFN-α-2a. PBMCs were isolated from patients prior to the daily chemotherapy on days 1, 2 and 4 of a 7 day course of IFN-α-2a, with 5-FU/LV given on days 2–6. (a) presents the pmol 5-FU catabolised per min per million cells (mean ± S.E.M., and represents 47 matched samples from all 3 days). (b) shows the absolute difference in the rate of 5-FU catabolism, determined by subtracting the baseline level from that on either day 2 or day 4 in matched blood samples from all available patient cycles (day 2, *n* = 54; day 4, *n* = 63). The differences in paired values were analysed by the Wilcoxon signed-rank test; the *P* values are shown. Adapted from [22].

5-FU AUC or *Cpss* (or decreased clearance) and more severe gastrointestinal toxicity in these studies suggests that the pharmacokinetic interaction of IFN-α and 5-FU contributed to the increased toxicity [17, 24]. A key issue is whether patients would be able to tolerate the same pharmacological exposures to 5-FU in the absence of IFN-α. While one might suppose the pharmacological interaction serves as a surrogate marker for a cellular effect of IFN-α pertaining to 5-FU, it also provides evidence that normal tissues are very sensitive to IFN-α. Non-selective enhancement of 5-FU toxicity may result in no net therapeutic gain.

The National Surgical Breast and Bowel Project (NSABP) is testing the contribution of IFN-α-2a to 5-FU/LV using the National Cancer Institute (NCI) regimen in the adjuvant treatment of Dukes' stage B and C colon cancer (protocol C–05). Accrual to this important clinical trial was completed in early 1994, with approximately 1000 patients per arm. The results of this NSABP trial should definitively answer whether the addition of IFN-α-2a to 5-FU/LV produces a therapeutic benefit in terms of disease-free and overall survival compared to 5-FU/LV alone.

Studies are in progress to correlate TS expression with clinical outcome in patients with advanced colorectal cancer treated with the 5-FU/LV/IFN-α-2a regimen. If the preliminary findings that TS content prior to therapy or the extent of TS inhibition following treatment predict clinical response can be confirmed, then it will raise the hope that TS-targeted therapy can be selected or avoided in patients according to the TS expression in their tumour tissue.

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