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ORIGINAL ARTICLE

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Effect of interferon on 5-fluorouracil-induced perturbations in pools of deoxynucleotide triphosphates and DNA strand breaks

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Abstract Interferon (IFN) augments the anabolism of 5-fluorouracil (5FU) to its active metabolite, fluorodeoxyuridylate (FdUMP), which inhibits thymidylate synthase (TS). We sought to determine whether this resulted in greater perturbations of nucleotide pools and if so, whether this was associated with an increase in cell lethality, specifically focussing on the lethal cellular lesion, DNA double strand breaks (dsb). To determine whether combination therapy with 5FU + IFN resulted in greater depletion of thymidine nucleotide pools than 5FU alone, a highly sensitive DNA polymerase assay was used. In two human colon cancer cell lines, treatment with 5FU + IFN resulted in a rapid decrease in levels of dTTP by 95%. The addition of IFN to 5FU resulted in greater depletion of dTTP levels over treatment with 5FU alone by up to fourfold, and markedly augmented the dATP/dTTP ratio. The addition of IFN to 5FU had no effect on 5FU-induced perturbations in dCTP, dGTP or dATP pools at 8 and 12 h. Measurement of DNA dsb demonstrated that treatment of HT-29 cells with 10 μM 5FU for 24 h did not increase DNA dsb versus control. The combination of 5FU + 500 U/ml IFN, however, resulted in an increased number of dsb versus both 5FU and untreated control cells ($P < 0.01$), equivalent to 0.74 ± 0.12 Gy. The addition of IFN to 5FU resulted in a selective further depletion of pools of dTTP and an increase in the number of DNA dsb versus 5FU treatment alone.

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Introduction

The combination of the fluorinated pyrimidine, 5-fluorouracil (5FU), and interferon (IFN) synergistically inhibits the growth of two human colon cancer cell lines, HT-29 and SW480, in vitro in a dose-dependent fashion [1]. In mouse and human adenocarcinoma cells and human HL-60 promyelocytic leukemia cells, this growth inhibitory effect can be reversed by exogenous thymidine [2, 3]. In HL-60 cells growth inhibition is associated with a decrease in thymidylate synthase (TS) activity [4] suggesting that inhibition of cell proliferation is mediated via inhibition of the 5FU target enzyme, TS. In HT-29 cells, IFN-α augments the anabolism of 5FU to its active metabolite, 5-fluorodeoxyuridinemonophosphate (FdUMP) by inducing the enzyme, thymidine phosphorylase [5]. As FdUMP bonds TS, covalently blocking binding sites for the endogenous substrate, deoxyuridylate (dUMP), these findings suggest that IFN may augment 5FU cytotoxicity by increasing inactivation of TS.

The precise mechanism whereby TS inactivation results in cell death is unknown. In a murine model, perturbations of nucleotide pools by thymidine depletion have been found to result in activation of an endogenous endonuclease which results in an increase in DNA double-strand breaks (dsb) [6]. In a TS-negative mutant of the same murine cell line, thymidine deprivation has the same effect [7]. Inhibition of TS may result in DNA dsb either through misincorporation of dUTP and/or misrepair of the uracil-containing lesion [8, 9]. Alternatively, 5FU treatment may result in DNA strand breaks by direct incorporation of FdUTP into DNA [10].

In order to determine whether IFN augments depletion of thymidine pools, we modified the DNA

polymerase assay developed by Sherman and Fyfe [11] to enhance its sensitivity and specificity. The selectivity of the IFN effects were gauged by examining effects on other dNTPs. Furthermore, to determine whether these perturbations result in enhanced DNA damage, DNA dsb were assayed. In this study we observed a selective and substantial reduction in cellular dTTP levels with 5FU treatment that was further potentiated by IFN, and an increase in DNA dsb with IFN treatment.

Materials and methods

Cell culture.

HT-29 and SW480 cells were a generous gift from Dr. Leonard Augenlicht. Cells were maintained in RPMI-1640 (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, N.Y.) and 1% penicillin-streptomycin at 37 °C in 5% CO_2. For drug exposures, cells were removed from standard culture medium 24 h prior to drug exposure and incubated in folate-free RPMI-1640 (GIBCO) with 10% dialyzed FBS (D-FBS) and either 0.5 μ*M* folic acid or 80- n*M* 5-CH_3-tetrahydrofolate. There were no differences in results with either folate.

Drugs and reagents

Recombinant interferon alfa-2a (IFN) was a gift from Hoffman-LaRoche (Nutley, N.J.). 5-Fluorouracil (5FU) was from Lyphomed (Rosemont, Ill.). DNA polymerase I, large fragment (Klenow fragment) and Dulbecco's phosphate-buffered saline (D-PBS) were from GIBCO. Oligonucleotides were from American Synthesis, Pleasanton, Calif.. All other reagents were from Sigma Chemical Co. (St. Louis, Mo.).

Radioisotopes

Thymidine 5′-triphosphate, tetrasodium salt, [methyl-^{3}H], 19 Ci/mmol, was from New England Nuclear (Boston, Mass.). [2, 8-^{3}H]deoxyadenosine triphosphate, 32 Ci/mmol, was from Moravek Biochemicals (LaBrea, Calif.).

Preparation of cell extracts for measurement of dNTP pools

Cells were incubated in folate-free RPMI-1640 with 10% D-FBS and 0.5 μ*M* folic acid with or without 5FU and/or IFN for various times in six-well Falcon tissue culture plates (Becton Dickinson, Lincoln Park, N.J.). The medium was rapidly poured off, and without washing [12] 200 μl ice-cold perchloric acid (PCA) was added and the mixture left for 30 min at 4 °C. The extract was centrifuged for 2 min at 12 000 *g*, then the supernatant was neutralized to pH 7.4 with 400 μl alamine/Freon as previously described [13]. Extracts were again centrifuged for 2 mins, and the top layer removed for the assay.

Measurement of dNTP pools

The DNA polymerase assay, modified from that of Sherman and Fyfe [11], was employed. Oligonucleotides were modified in order to increase the sensitivity and decrease the background of the assay. The template sequences employed were:

dNTP	Sequence
dTTP	5′TTT ATT TAT TTA TTT ATT TAG GCG GTG GAG GCG G-3′
dCTP	5′TTT GTT TGT TTG TTT GTT TGA AAG GTG GAG GCC C-3′
dATP	5′AAA TAA ATA AAT AAA TAA ATG GCG GTG GAG GCG G-3′
dGTP	5′TTT CTT TCT TTC TTT CTT TCG GCG GTG GAG GCG G-3′

The primer sequences were:

dNTP	Sequence
dCTP	5′GGG CCT CCA CCT TT-3′
dTTP, dATP, dGTP	5′CCG CCT CCA CCG CC-3′

Oligonucleotides were dissolved in TE buffer (10 m*M* Tris HCl, pH 7.4, 1 m*M* EDTA), and their concentrations were confirmed by spectroscopy. Templates and primers were mixed in TE buffer, vortexed, heated to 65 °C for 20 min and cooled to room temperature. Samples were diluted in water to 0.8 μ*M* and stored at -20 °C.

The assay mixture included 10–67 μl cell extract (depending on the particular nucleotide being measured), 5 m*M* $MgCl_2$, 100 m*M* Hepes, pH 7.4, [2, 8-^{3}H]deoxyadenosine triphosphate or 0.65 μCi [methyl-^{3}H]thymidine 5′-triphosphate, tetrasodium salt, 4 p*M* oligonucleotide, 0.065 *U* and Klenow fragment, in a total volume of 100 μl. The mixture was vortexed for 10 s, then incubated at 37 °C for 1 h. Assay mixture (30 μl) was spotted on DE81 filters (Whatman, Hillsboro, Ore.) prewetted with 1% sodium pyrophosphate/5% trichloroacetic acid and washed with 20 ml of the same solution three times. Filters were then dried and counted by liquid scintigraphy. Assays were performed in replicates of six, and repeated three times. dNTP standards were employed for each experiment; standard curves were linear to 0.125 pmol and correlation coefficients > 0.99 were obtained routinely. The assay was linear with respect to time and concentration of dNTP. Isotopic dilution by unlabelled, endogenous nucleotide was estimated for each assay. This was considered negligible, as there was always at least a more than tenfold excess of exogenously added nucleotide per assay tube.

Preparation of agarose plugs for PFGE

HT-29 human colon carcinoma cells were seeded in 25-cm^3 flasks (Corning, Corning, N.Y.) in 5 ml medium composed of RPMI-1640, 10% FBS, 1% 1 × penicillin-streptomycin-neomycin (PSN) antibiotic mixture (GIBCO) such that at time of harvest each flask contained approximately 5×10^6 cells in the logarithmic growth phase. The medium was changed 2 days prior to harvest to 2 ml RPMI-1640, 10% dialyzed DFBS, 1% PSN with 2 μCi/ml [methyl-^{3}H] thymidine (740 GBq/mmol, 20.0 Ci/mmol) (NEN, Boston, Mass.). In early experiments, a separate flask of cells was also labelled with [methyl-^{14}C] thymidine for use as an internal control, but this was discontinued when it was noted that all lanes ran essentially identically. After 24 h, the medium was changed to 2 ml RPMI-1640 folate-free, 10% D-FBS, 1% PSN with 80 n*M* 5-methyltetrahydrofolate (Sigma, St. Louis, Mo.). For drug-treated samples, 5FU (Solopak, Elk Grove Village, Ill.) was added to produce a final concentration of 10 μ*M* with or without 500 U/ml IFN alfa-2a (Hoffman-LaRoche, Nutley, N.J.) for 24 h.

After 24 h, cells were released from the flasks with 1 × trypsin-EDTA. Trypsin was neutralized with FBS-containing medium. The flasks were gently scraped, and the cells were pelleted in 15-ml conical tubes (Becton Dickinson, Oxnard, Calif.) at 900 rpm for 5 min at 4 °C. The supernatant was decanted and the insides of the

conical tubes wiped to remove excess moisture. 45 µl of L buffer (50 m*M* EDTA; 10 m*M* Tris, pH 8; 20 m*M* NaCl) were added to each pellet [14]. 60 µl of 1.2% agarose for pulsed field gel electrophoresis (PFGE) sample preparation (Sigma) in 2 × TAE, were added to each pellet and gently mixed using pipets with their tip cut off. Cell mixture (90 µl) was then placed into a plug mold (Biorad). Plugs were cooled at 4 °C for 5 min and then extruded into 50-ml conical tubes (Becton Dickinson). 0.5 ml of digestion buffer (100 m*M* EDTA, pH 8; 0.2% sodium deoxycholate; 1% sodium lauryl sarcosine; 1 mg/ml proteinase K) per plug, was added to each tube. The plugs were allowed to digest for 24 h at 50 °C. The digestion buffer was drained and the plugs washed four times with wash buffer (20 m*M* Tris, pH 8, 50 m*M* EDTA) for 30 min at room temperature without agitation. The plugs were stored in fresh buffer at 4 °C until use (< 1 week).

Irradiation of plugs

Cell blocks of non-drug-treated cells were irradiated at room temperature in wash buffer using 0.66 MeV photons from a ^{137}Cs source (Picker International, Cleveland, Ohio). Irradiated plugs were used to quantitate the amount of DNA dsb and to generate a standard curve. Radiation doses of 2.5–10 Gy were delivered at a rate of 60 Gy/min, and doses of 0.5–1.0 Gy were delivered at a rate of 1.1 Gy/min.

PFGE

Induction of DNA dsb was measured by PFGE of treated and untreated cells [16–18]. Chromosomal grade agarose (150 ml, 0.6%; Biorad, Hercules, Calif.) in 1 × TAE was prepared. Radiation-, drug-treated, and control plugs were inserted into the running gel. *Schizosaccharomyces pombe* chromosomes (Biorad) were employed as size markers. Plugs were sealed with 0.6% low melting point agarose (Sigma). The gel was then run in a CHEF-DR II (Biorad) PFGE apparatus for 72 h at 2 V/cm with a 30-min switch interval in 1 × TAE buffer at 14 °C. The gel was stained with ethidium bromide and photographed. The gel was then sliced into lanes, and the lanes into 5-mm slices. Each slice was then melted in 50 µl 1 *N* HCl for 10 min. The melted slices were neutralized in 50 µl 1 *N* NaOH and counted in 5 ml scintillation fluid (RPI, Mount Prospect, Ill.) on a 1209 Rackbeta scintillation counter (LKB Wallac, Turku, Finland). Slices were counted until counts fell below four times background; measurements of total radioactivity before gel electrophoresis and afterwards indicated that > 99.9% of all radioactivity was accounted for by this method.

Data Analysis

All drug treatments were repeated in replicates of at least 12. Radiation treatments used for the development of a standard curve were performed two to six times. Untreated control plugs were included on all gels so that results could be corrected for breakage due to processing and handling of the DNA. For drug-treated samples, averages are expressed as the mean +/− the standard error.

Breakage patterns were analyzed by the measurement of the cumulative percentage of tritium-labelled DNA which entered the running gel matrix with successive slices minus that of the untreated control on that gel. Comparisons were made using the repeated measurement method. Using log-linear regression analysis, a radiation standard curve of the total corrected percentage of tritiated DNA entering the matrix versus the radiation dose was developed. For drug-treated samples, a radiation equivalent was estimated using the radiation standard curve. The calculation of dsb frequency of radiation doses was performed using the method described by Blocher [19].

Results

Effects of 5FU/IFN treatment on pools of dNTPs

As shown in Fig. 1, treatment with 100 µ*M* 5FU and 500 U/ml IFN resulted in profound changes in the composition of nucleotide pools in both SW480 and HT29 cells. The most important change in both cell lines was the depletion of pools of dTTP beginning at 2 h, with 95% depletion of dTTP pools by 8 h. Despite continued exposure to drug, dTTP levels recovered partially (SW480) or almost completely (HT-29) by 12–24 h. Pools of dATP were also affected, with a rapid rise in SW480 cells, reaching a 12-fold increase by 8 h and persisting at elevated levels for 24 h. Effects on dATP pools in HT-29 cells were smaller with only a two-fold increase at 2 h followed by a decline in levels from 8 to 24 h.

Effects on other dNTPs were less pronounced. Pools of dGTPs were depleted in both cell lines with a pattern similar to, but less thorough than, dTTP. Pools of dCTP rose slightly in both cell lines. A similar pattern of effects was observed in cells treated with 10 µ*M* 5FU and 500 U/ml IFN (data not shown).

Effects of IFN on 5FU-induced changes in nucleotide pools

To determine the contribution of IFN to the changes in composition of deoxynucleotide pools, cells were incubated with 5FU with or without IFN for 8 and 12 h, the time-points at which maximal changes in pool sizes had been observed. IFN alone had no effect on levels of

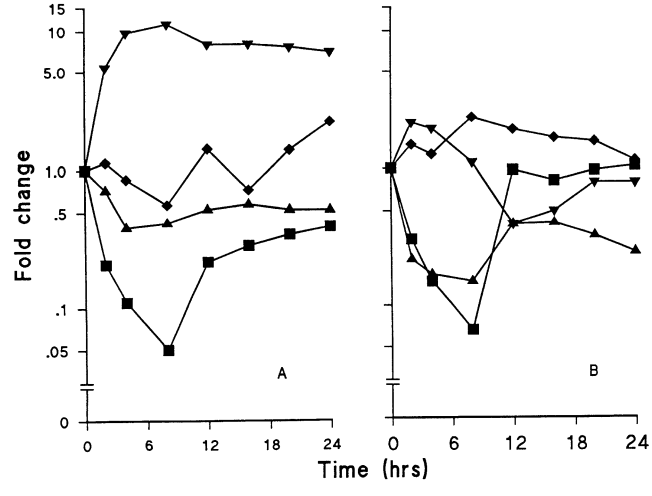

Fig. 1A, B Effect of treatment with 5FU + IFN on dNTP pools in SW480 (**A**) and HT-29 (**B**) cells from 0 to 24 h. Cells were incubated with 100 µ*M* 5FU + 500 U/ml IFNα, and at 2, 4, 8, 12, 16, 20 and 24 h were assayed as described in Methods (▼ dATP, ◆ dCTP, ▲ dGTP, ■ dTTP). Each value is the mean of six replicates; standard error bars lie within the points

Table 1 Effect of 100 μ*M* 5FU ± 500 U/ml IFN on dNTP pools at 0, 8 and 12 h

Cell type		0 h	8 h	12 h
dTTP (pmol/10^6 cells)				
HT-29	− IFN	65.71 ± 4.65	11.92 ± 1.50	67.60 ± 5.81
	+ IFN	–	7.52 ± 0.50*	66.75 ± 6.41
SW480	− IFN	55.00 ± 3.55	8.31 ± 2.06	19.46 ± 0.61
	+ IFN	–	2.46 ± 0.32*	13.88 ± 1.52*
dCTP (pmol/10^6 cells)				
HT-29	− IFN	37.05 ± 1.08	21.15 ± 2.61	47.04 ± 2.60
	+ IFN	–	22.47 ± 1.68	43.69 ± 2.82
SW480	− IFN	28.08 ± 1.36	15.66 ± 2.70	18.72 ± 1.26
	+ IFN	–	19.80 ± 1.08	22.14 ± 1.44
dGTP (pmol/10^6 cells)				
HT-29	− IFN	5.44 ± 0.09	1.08 ± 0.08	2.55 ± 0.11
	+ IFN	–	1.05 ± 0.09	2.51 ± 0.16
SW480	− IFN	5.09 ± 0.16	0.65 ± 0.04	1.01 ± 0.05
	+ IFN	–	0.77 ± 0.03	0.89 ± 0.05
dATP (pmol/10^6 cells)				
HT-29	− IFN	7.75 ± 0.71	12.61 ± 0.64	2.27 ± 0.35
	+ IFN	–	12.23 ± 0.47	3.25 ± 0.21
SW480	− IFN	8.75 ± 1.10	13.13 ± 0.88	14.46 ± 1.42
	+ IFN	–	15.48 ± 0.48	15.83 ± 0.78

*$p < 0.03$

dNTP pools. As shown in Table 1, when employed in combination with 100 μ*M* 5FU, IFN treatment resulted in a significant enhancement of 5FU effects on dTTP pool depletion at 8 and 12 h in SW480 cells and at 8 h in HT-29 cells. In SW480 cells at 8 h, treatment with IFN + 5FU resulted in a 70% decrease in dTTP levels over those achieved with 5FU alone, while in HT-29 cells, levels of dTTP decreased by 37% at 8 h in 5FU/IFN-treated cells versus those treated with 5FU alone. IFN also significantly enhanced depletion of dTTP pools by 10 μ*M* and 1 μ*M* 5FU, in HT-29 cells (data not shown) at 8 h. IFN did not augment 5FU-induced depletion of dTTP pools at time-points earlier than 8 h and had no effect on pools of other dNTPs.

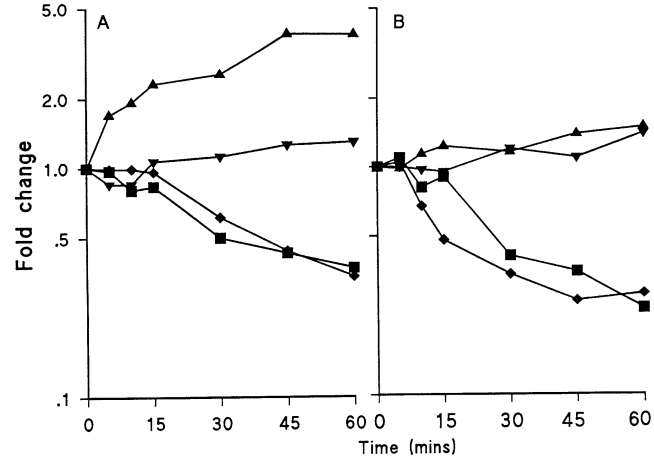

Fig. 2A, B Effect of treatment with 5FU + IFN on dNTP pools in SW480 (**A**) and HT-29 (**B**) cells from 0 to 60 min. Cells were incubated with 100 μ*M* 5FU + 500 U/ml IFNα, and were assayed for dNTP levels at various time points as described in Methods (▲ dATP, ▼ dCTP, ■ dGTP, ◆ dTTP). Each value is the mean of six replicates; standard error bars lie within the points

Early effects of 5FU/IFN on dNTP pools

As shown in Fig. 2, the effects on pools of dNTPs were observed at early time-points, within 1 h of starting treatment. In both cell lines, pools of dTTP and dGTP were decreased by about 75% at 1 h. Pools of dATP rose sharply in SW480 cells, but not in HT-29 cells, by four-fold. Pools of dCTP were unaffected.

Effects of 5FU/IFN on DNA double-strand breaks

Radiation treatment of HT-29 cells embedded in agarose plugs with 0.5–10 Gy resulted in a log-linear increase in DNA dsb (Fig. 3A). Treatment of HT-29 cells with 10 μ*M* 5FU for 24 h resulted in no increase in DNA dsb as compared with untreated control cells. In contrast, treatment of HT-29 cells with 10 μ*M* 5FU + 500 U/ml IFN resulted in a significant increase in DNA dsb versus cells treated with 5FU alone ($P < 0.01$) and versus untreated controls ($P < 0.01$; Fig. 3B). Calculation of Gy equivalents employing log-linear regression analysis demonstrated that treatment with 5FU + IFN released amounts of DNA into the gel equivalent to the effects of 0.74 ± 0.12 Gy (Fig. 3A).

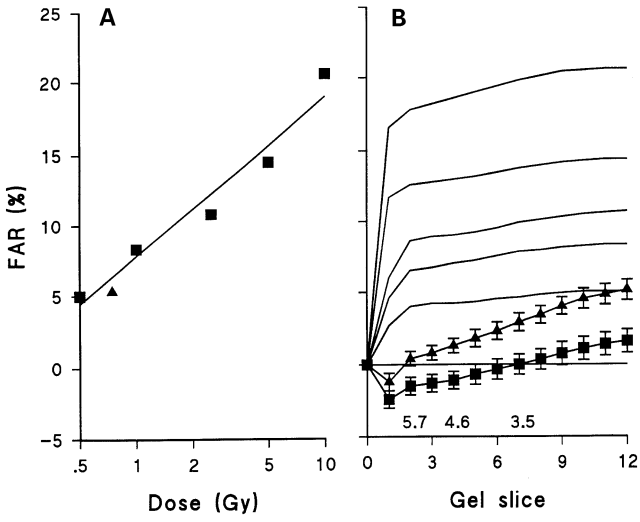

Fig. 3A, B Induction of DNA dsb by γ-radiation or 5FU ± IFN in HT-29 cells. **A** The fraction of activity released (FAR) was calculated as the amount of radiolabel released into the gel at each slice as a proportion of total radioactivity taken up by the cells. The total FAR for each dose of γ-radiation is plotted versus the log of the radiation dose (■) ($r = 0.9771$, $r^2 = 0.9547$, $P = 0.004$). Treatment with 10 μ*M* 5FU + 500 U/ml IFN for 24 h (▲) resulted in the equivalent of 0.74 ± 0.12 Gy. **B** The FAR for successive slices from gels containing either radiated cells (*solid lines*), cells treated with 10 μ*M* 5FU (■), or 5FU + IFN (▲). Each value is the mean of six experiments (*bars* SEM). Radiation doses (Gy): 0, 0.5, 1, 3, 5, 10. The results are corrected for background DNA fragmentation. *S. pombe* markers (Mb) are indicated by numbers 3.5, 4.6 and 5.7. 5FU + IFN treatment differs from both control and 5FU alone ($P < 0.01$ for both)

Using the formula of Blocher [19], this is equivalent to $1.0 \pm 0.2 \times 10^{-8}$ dsb/bp. Nevertheless, the pattern of DNA breakage observed clearly differed from that produced by γ-radiation (Fig. 3B) with a shifting of DNA fragment size to smaller (< 5.7 Mb), fragments, suggesting that the number of dsb may be substantially higher. The linear trend for cells treated with 5FU + IFN differed from that of radiated cells ($P < 0.005$).

Discussion

In this study we demonstrated that IFN augmented the 5FU-induced depletion of dTTP pools at 8–12 h, and that this was associated with an increase in DNA dsb with the production of large, Mb DNA fragments. This effect appeared to be selective for dTTP as IFN treatment in combination with 5FU did not affect pools of dCTP, dATP or dGTP beyond those changes observed with 5FU alone.

The enhancement of 5FU-induced depletion of dTTP pools by IFN was modest relative to the baseline effects of 5FU alone. Nevertheless, the absolute magnitude of the effects may not be the most significant factor. For example, Yoshioka et al. [6] have postulated that it is the change in the ratio of dATP to dTTP, rather than thymidine-less stress per se, that triggers endonuclease activity and an increase in DNA strand breaks. In contrast Keyomarsi and Moran [20] have observed, with respect to fluoropyrimidine-induced thymidine depletion and cell death in the L1210 mouse leukemia tumor model, that > 80–90% inhibition of preexisting TS is necessary in order to have *any* effect on the growth of L1210 cells, and presumably, a much higher degree of inhibition of TS would be required for complete inhibition of growth, especially in the context of a fivefold increase in TS expression following exposure to fluoropyrimidines. Our studies support the view that IFN augments an imbalance in the dATP/dTTP ratio by up to fourfold at 8 h. However, it is not clear whether it is the depth of the depression of dTTP levels above that obtained with 5FU alone or the dATP/dTTP ratio that is the critical effect.

The depletion of dTTP pools observed in these cell lines was likely due to enhanced inhibition of TS by IFN. In fluoropyrimidine-treated cells, IFN augments inhibition of TS by dual and complementary mechanisms. First, IFN induces thymidine phosphorylase expression and activity, the rate-limiting step in anabolism of 5FU to its active metabolite, FdUMP, both in vitro and in vivo in patients treated with 5FU + IFN [21, 22]. This results in a significant increase in FdUMP levels in HT-29 cells in vitro [5]. Of interest, thymidine phosphorylase activity in tumor cells may be higher than in surrounding normal tissues, conferring a degree of selectivity for this effect [23]. Second, IFN-γ abrogates 5FU-induced induction of TS in vitro [4, 24, 25]. Inhibition of TS activity may have resulted from direct inhibition of translation of TS by IFN-γ [26] affecting pathways that have only recently been defined [27, 28]. Furthermore, IFN in vitro inhibits thymidine salvage pathways [5]. All of these factors may be contributory to the decline in dTTP levels observed.

Houghton et al. [29] have also demonstrated that the addition of IFN to 5FU treatment in vitro results in an increase in single- and double-strand DNA breaks in GC_3/cl cells. These effects are dose- and schedule-dependent, and are enhanced by the addition of the reduced folate, leucovorin (LV). For example, treatment with 1 μ*M* 5FU is equivalent to treatment with 2.5 Gy, with 5FU + IFN-α, equivalent to 4.5 Gy, and with 5FU + IFN-α + LV, equivalent to 6 Gy, effects somewhat higher than observed in our studies, possibly because of the different methodology for calculation of radiation effects or the higher concentrations of IFN employed. Dusenbury et al. [17] employed a pulsed-field gel method similar to that employed in our studies to compare DNA dsb induced by FdUrd with those induced by radiation treatment in HT-29 cells. As in our hands, they observed comparable levels of DNA released into the gel (25–30%) with either drug or radiation treatment, but a markedly different electrophoretic pattern, suggesting that "double strand

breaks caused by FdUrd are not located randomly with respect to the entire genome," as opposed to the radiation-induced breaks. Based on our data, we are only able to report a difference in the electrophoretic patterns, but cannot definitively conclude that one type of break is non-random.

Ours is the first report associating augmented perturbation of nucleotide pools by 5FU + IFN with an increase in DNA dsb. The precise relationship between these events requires further study. One likely relationship is that IFN enhancement of 5FU-induced dTTP pool depletion results in further elevation of the dUTP/dTTP ratio, with increased misincorporation of dUTP into genomic DNA, and increased levels of DNA excision/repair [30]. Intracellular dUTP incorporation has been shown to be directly proportional to DNA damage and growth inhibition in A549 lung carcinoma cells treated with the TS inhibitor, CB3717 [9], and cycles of futile repair and excision associated with dUTP incorporation have been associated with increased levels of DNA strand breaks [31, 32]. Alternatively, in the murine FM3A model, imbalances in pools of dNTPs have been associated with activation of an endogenous endonuclease with an increase in DNA dsb and the production of small DNA fragments, 100–200 kbp [6, 7, 33]. These fragments are substantially smaller than the Mb fragments we observed in our studies, casting doubt on whether this represents the same mechanism. Others have drawn attention to the potential role of IFN-induced cell cycle effects in induction of DNA damage [34], with particular attention to the association of thymine-less stress and the role of cell cycle checkpoints [35].

We conclude that IFN selectively augments 5FU-induced depletion of dTTP pools by enhancing thymidine phosphorylase activity and/or inhibiting TS activity, and that this results in an increase in DNA dsb, most likely through misincorporation of dUTP and the action of uracil-DNA glycosylase. As *any* increase in dsb can be a lethal event, this is a likely explanation for the increase in cytotoxicity observed with the combination of 5FU + IFN. Further studies are required to focus on the precise mechanism by which this occurs and to assess whether these dsb differ from the random breaks associated with ionizing radiation.

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