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Interferon augments the cytotoxicity of hydroxyurea without enhancing its activity against the M2 subunit of ribonucleotide reductase: effects in wild-type and resistant human colon cancer cells

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Abstract The effects of prolonged exposure to the ribonucleotide reductase (RR) inhibitor, hydroxyurea (HU), were assessed in the presence or absence of recombinant interferon alfa-2a (IFN) in wild-type human colon cancer cells (HT-29) and variants expressing low-level resistance to HU (R200). IFN at nontoxic concentrations decreased the IC_{50} of HU from 368 μM to 215 μM ($P < 0.01$) in wild-type cells, but not in the resistant variants. Potential cellular targets for the HU/IFN interaction were examined. In wild-type, but not resistant cells, treatment with HU at clinically achievable concentrations (1000 μM) resulted in rapid early inhibition of RR activity between 4 and 24 h after treatment with a maximal decrease of 65% at 12 h, decreases in cellular levels of dATP, dCTP and dGTP by 50–90% over the same time course, and a two- to fourfold increase in the level of mRNA for both the M1 and M2 subunits of RR, at 24, but not between 1 and 4 h, which probably represents a response to the earlier decrease in RR activity. IFN at a clinically achievable concentration (500 U/ml) failed to augment the effects of HU on RR protein, RR mRNA levels or RR enzyme activity in either the wild-type or resistant cells, suggesting that the mechanism by which IFN augments the effects of HU in the wild-type cells is independent of the effects of HU on M2.

Key words Interferon alfa-2a · Hydroxyurea · Rbonucleotide reductase · Drug resistance · Colon cancer

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Introduction

Hydroxyurea (NSC 32065, HU) selectively inhibits DNA synthesis without affecting the synthesis of proteins or ribonucleic acid in vitro in tumor cell lines. HU has been widely employed in the treatment of chronic myelogenous leukemia and the myeloproliferative syndromes, and has also demonstrated efficacy as a radiation sensitizer in the treatment of locally advanced carcinoma of the cervix. While occasional reports of activity have been noted in the treatment of solid tumors, the use of HU has been minimal (reviewed in reference 1).

HU acts by inhibition of ribonucleotide reductase (RR), an enzyme that ensures a balanced supply of nucleotides for DNA synthesis (reviewed in reference 2). In eukaryotes, RR is a 258 kDa protein with an $\alpha_2\beta_2$ subunit structure with α_2 characterized as either R1 (in *E. coli*) or M1 (in eukaryotes) and β_2 as either R2 or M2. M1 is the larger subunit, containing the substrate and effector binding sites, and M2 is the smaller subunit, containing a unique iron tyrosyl radical, Tyr^{22}, and an oxygen-linked iron (FeIII) center, which generates and maintains the tyrosyl radical. HU acts as a radical scavenger, inactivating the tyrosyl radical on M2, thus inhibiting the ability of the enzyme to reduce ribonucleotide diphosphates to their deoxy analogues.

As expected with an inhibitor of DNA synthesis, the activity of HU is highly S-phase specific [3]. Furthermore, the target of HU activity, M2, has a short half-life and can be rapidly regenerated in vivo. Thus, HU would be more likely to have activity when employed in a prolonged, continuous fashion. Oral administration of HU, the current standard practice, is thus disadvantageous. In detailed pharmacokinetic studies [4] oral administration of 800 mg/m^2 HU every 4 h for 18 doses resulted in peak levels of 800 μM (700–900 μM), 1080 μM (770–1250 μM), and 2480 μM (1550–3200 μM) after dose 1,7 and 13 respectively. However,

trough levels were only 410 μ*M* (290–530 μ*M*), 730 μ*M* (680–770 μ*M*) and 550 μ*M* (450–650 μ*M*) after the same doses. In contrast, studies of 72-h parenteral HU administration by the same investigators revealed essentially steady-state plasma levels for 72 h at 488 μ*M*, 460 μ*M*, 537 μ*M*, 760 μ*M*, and 1090 μ*M* for doses of HU from 2–3 mg/m^2 per min (2.9–4.3 g/m^2 per day). Thus, the failure of earlier HU regimens employing oral administration may have resulted at least in part from the lack of consistent plasma levels of the drug, thus allowing recovery of cellular RR activity from the inhibitory effects.

In 1993, parenteral HU, which had been previously studied [5, 6], but which had never been widely employed in clinical practice, became available for phase I and II clinical trials. A phase I trial of HU administered as a long-term infusion in combination with the fluorinated pyrimidine, 5-fluorouracil (5FU), and the negative growth regulator, interferon-α (IFN), was initiated at our institution to assess the maximum duration for which HU could be administered in such a regimen. To better understand the mechanism by which HU acts during prolonged, continuous exposure in the presence and absence of IFN, in vitro studies employing human colon cancer cell lines were undertaken. In addition, to determine the mechanism by which acquired resistance to HU develops, we have developed and studied variant cell lines grown continuously in HU which exhibit low-level resistance.

Material and methods

Cell culture

HT-29 cells were maintained in RPMI-1640 (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin at 37 °C in an atmosphere containing 5% CO_2. HT-29 R200 cells were grown in the same medium with 200 μ*M* HU present continuously. 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 0.5 μ*M* folic acid without antibiotics.

Drugs and reagents

Recombinant interferon alfa-2a (IFN) was a gift from Hoffman LaRoche (Nutley, N.J.). 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., and the Oligonucleotide Facility at the Albert Einstein Cancer Center. All other reagents, including HU, were from Sigma Chemical Co. (St. Louis, Mo.).

Radioisotopes

Thymidine 5′-triphosphate, tetrasodium salt, [methyl-^{3}H] (19 Ci/mmol) and [α-^{32}P]deoxycytidine 5′-triphosphate, tetra(triethylammonium salt) (3000 Ci/mmol) were from New England Nuclear (Boston, Mass.). [2,8-^{3}H]deoxyadenosine triphosphate (32 Ci/mmol) and cytidine 5′-diphosphate, [U-^{14}C] (400 uCi/mmol) were from Moravek Biochemicals (LaBrea, Calif.).

Measurement of cell growth

Cell proliferation was estimated using a slight modification of the sulforhodamine B (SRB) assay [7]. Results obtained with this assay were similar to those obtained with a clonogenic assay as previously reported [8].

Flow cytometry

HT-29 cells were treated with 1000 μ*M* HU for various times, then analyzed by flow cytometry by a modification of the method of Vindelov et al. [9] as previously described [10].

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 HU 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 [11] 200 μl ice-cold perchloric acid (PCA) was added and left for 30 mins. The extract was centrifuged for 2 min at 12 000 *g*, and the supernatant was then neutralized to pH 7.4 with 400 μl of alamine/ Freon as previously described [12]. Extracts were again centrifuged for 2 min, and the top layer removed for the assay.

Measurement of dNTP pools

The DNA polymerase assay, modified from that of Sherman and Fyfe [13], was employed as previously described [8]. 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 G3′
dCTP	5′TTT GTT TGT TTG TTT GTT TGA AAG GTG GAG GCC C3′
dATP	5′AAA TAA ATA AAT AAA TAA ATG GCG GTG GAG GCG G3′
dGTP	5′TTT CTT TCT TTC TTT CTT TCG GCG GTG GAG GCG G3′

The primer sequences were:

dNTP	Sequence
dCTP	5′GGG CCT CCA CCT TT3′
dTTP, dATP, dGTP	5′CCG CCT CCA CCG CC3′

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, 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 thymidine 5′ triphosphate, tetrasodium salt [methyl-^{3}H], 0.65 μCi, 4 p*M* oligonucleotide, and 0.065 U 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 onto DE81 filters (Whatman, Hillsboro, Ore.) prewetted with 1% sodium pyrophosphate: 5% trichloroacetic acid and washed three times with 20 ml of the same solution. Filters were then dried and counted by liquid scintigraphy. Assays were performed in replicates of six. dNTP standards were employed for each experiment. Standard curves were linear to 0.125 pmol and correlation coefficients of ≥ 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 labelled over unlabelled nucleotide per assay tube.

Measurement of M1 and M2 by immunoblot

Cells were exposed to drug for various times as described above, washed and sonicated for 5 s three times on ice at 30% output in lysis buffer (10 m*M* Tris-HCl, pH 6.8; 1 m*M* dithiothreitol (DTT); 0.2 m*M* phenylmethylsulfonyl fluoride; 10 ng/ml aprotinin). Triton X-100 was added to a final concentration of 0.1% and the samples chilled for 15 min. Supernatants were isolated after centrifugation at 14 000 rpm for 15 min. Samples were centrifuged over a Millipore Ultrafree MC low-binding cellulose filter (30 000 NMWL, Millipore, Bedford, Mass) at < 7000 rpm for 30 min. A 20-μg sample of protein was suspended in sample buffer (62.5 m*M* Tris HCl, pH 6.8; 1% SDS; 0.01% bromphenol blue, 10% glycerol, 25 m*M* β-mercaptoethanol) and heated to 90 °C for 3 min. Samples were electrophoresed on an 8% polyacrylamide gel for 1 h at 30 mA in running buffer (25 m*M* Tris HCl, pH 8.0; 193 m*M* glycine; 0.1% SDS). Samples were transferred to a polyvinylidine difluoride membrane (Schleicher and Schuell), and soaked in blocking solution (GIBCO) at room temperature for 1 h. Blots were incubated with 1μg antibody to M1 (AD203, InRo Biomedtek, Umea, Sweden) [14] or 2 μg antibody to M2 (the monoclonal rat antitubulin antibody, YL1/2, Accurate Chemical, Westbury, N.Y.) [15], for 3 h at 4 °C. Blots were placed in TBST buffer (50 m*M* Tris base; 200 m*M* NaCl; 0.05% (v/v) Tween 20; GIBCO), then incubated with 2 μg of the appropriate biotinylated second antibody for 30 min and washed again with TBST. Blots were incubated with 0.3 μg/ml streptavidin-alkaline phosphatase (GIBCO) for 30 min at room temperature, then rewashed four times in TBST. Protein was detected by the addition of Lumi Phos (GIBCO), 1–2 ml/blot, followed by exposure to Kodak X-omat film for 3–5 h at room temperature. The authenticity of the bands was confirmed by comparison with recombinant mouse R1 ribonucleotide reductase subunit (kindly supplied by Dr. Lars Thelander, University of Umea, Sweden), which shares 97% homology with the human subunit [16].

Estimation of RR enzyme activity

RR activity was assayed by measuring the conversion of [^{14}C]cytidine 5′-diphosphate to [^{14}C]deoxycytidine 5′-diphosphate employing the method of Steeper and Steuart [17] as modified by Grill and Cheng (personal communication). Following drug treatment, cells were washed, suspended in extraction buffer (50 m*M* HEPES, pH 7.5; 1 m*M* $MgCl_2$; 2 m*M* DTT), and lysed with 30 strokes of a Dounce homogenizer. Following centrifugation at 14 000 rpm for 15 min, the supernatants were dialyzed overnight against 100 vol × 2 of extraction buffer at 4 °C. The CDP mix included: 0.1 *M* HEPES, pH 7.5; 10 m*M* DTT; 20 m*M* $MgCl_2$; 17 m*M* ATP; 0.5 m*M* CDP; 0.02 μCi [^{14}C]CDP. A mixture of 6 μl of the reaction mix, 4 μl DDH_2O, and 10 μl extract was incubated for 45 min. The reaction was stopped by boiling for 3 min. Snake venom phosphodiesterase (20 μl) was added and the mixture reacted for 2.5 h at 37 °C. The reaction was terminated by boiling for 3 min, and 200 μl DDH_2O was added. Following centrifugation at 16 000 rpm for 4 min, the supernatant was applied to a 0.2-ml borate column, and washed with 1 ml DDH_2O. The eluant was added to scintillation fluor and counted by liquid scintigraphy. Controls were identical except for the absence of $MgCl_2$ and ATP and the presence of 10 m*M* EDTA in the reaction mixture (which gave equivalent results to boiling the extract for 10 min).

Measurement of M1 and M2 RNA by Northern analysis

Cells were treated with HU with or without IFN, or with neither, for various times. RNA was isolated either in guanidine isothiocyanate (GIT) buffer layered over a cesium chloride cushion [18] or in a single-step procedure employing a monophasic solution of phenol and GIT (TRIzol Reagent, GIBCO), which gave equivalent results. Northern analysis was performed on an agarose-formaldehyde gel. Blots were transferred to nylon membranes and probed with ^{32}P-labelled probes specific for either M1 or M2 [19].

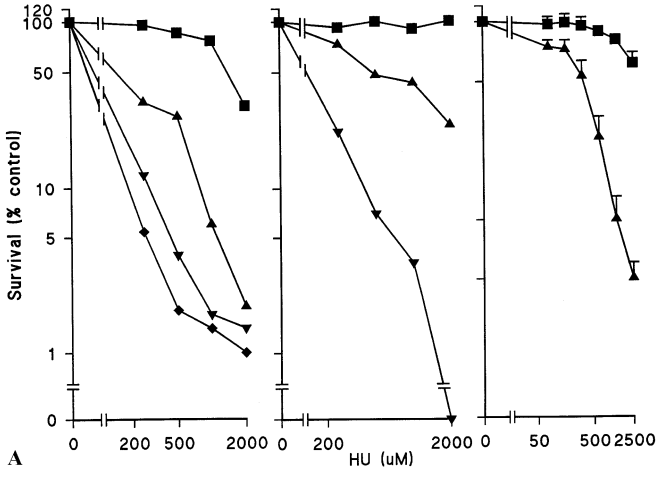
Results

Effect of duration of exposure on cytotoxicity of HU

As shown in Fig. 1A (left panel), treatment of HT-29 cells with HU resulted in exponential cell killing at concentrations of 200–2000 μ*M* for drug treatments longer than 24 h. Cell cycle analysis by flow cytometry confirmed the observation that duration of exposure was a critical determinant for cytokinetic effects (Fig. 1B) with progressive accumulation of cells in S-phase resulting from the longer exposures, but also demonstrating that the decrease in cell number at durations of HU exposure < 36 h, resulted primarily from a decrease in cell survival. As shown in Fig. 1A (center panel), IFN alone was nontoxic to cells at concentrations of 500–4000 U/ml for 36 h. In combination with HU, however, there was a decrease in the IC_{50} for HU following a 36-h exposure from 368 μ*M* to 215 μ*M* ($P < 0.01$), indicating that IFN at nontoxic concentrations augments the cytotoxic effects of HU against wild-type HT-29 cells. The HU-resistant R200 cell line exhibited resistance to HU (Fig. 1A, right panel) with an $IC_{50} > 2500$ μ*M* following a 36-h incubation with HU versus 368 μ*M* for the wild-type cells. IFN treatment did not augment the cytotoxicity of HU against the R200 variants.

Effects of HU + IFN on RR activity in sensitive and resistant cell types

RR activity was nearly twofold higher in resistant cells than in sensitive cells: 10.7 ± 0.26 v 5.5 ± 0.38 pmol/mg per min ($P < 0.0001$). Wild-type HT-29 cells treated



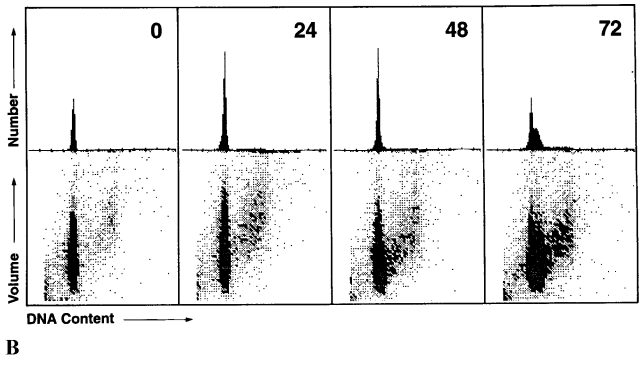


Fig. 1A Effects of HU on cell survival and proliferation. *Left panel* Prolonged exposures to HU resulted in greater cytotoxic and cytokinetic effects. HT-29 cells were exposed to 250–2500 μ*M* HU for 24 (■), 48 (▲), 72 (▼), or 96 (◆) h. Increasing exposure times resulted in reductions in the IC_{90} from > 2000 μ*M* to 850 μ*M*, 340 μ*M*, and 215 μ*M*. Each value is the mean of three experiments *bars* standard errors. *Center panel* Treatment with HU + 500 U/ml IFN for 36 h (▼) resulted in greater cell kill than treatment with HU alone (▲). (■) Treatment with IFN at 4000, 2000, 1000, and 500 U/ml for 36 h resulted in no decrease in cell number in the absence of HU. *Right panel* R200, HU-resistant variants of HT-29, express low-level resistance to HU. R200 (■) or HT-29 (▼) cells were exposed to HU for 36 h. Each values is the mean of three experiments, (*bars* standard errors). **B** HT-29 cells were treated with 1000 μ*M* HU for 0, 24, 48, or 72, then analyzed by flow cytometry. *Top panels* cell number v DNA content; *bottom panels* cell volume v DNA content. The progressive accumulation of cells in S-phase correlates with the duration of exposure to HU

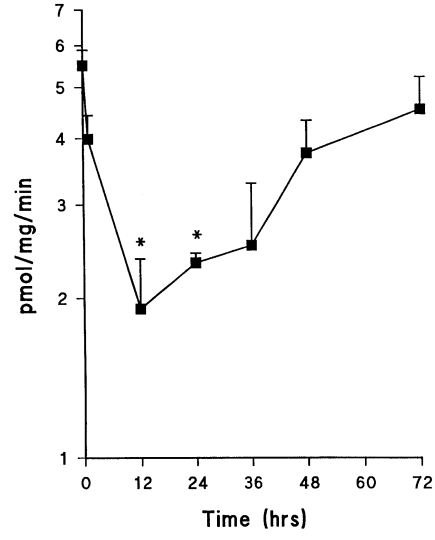


Fig. 2 Effects on RR activity in HT-29 cells. HT-29 cells were incubated with 1000 μ*M* HU for 4–72 h. Conversion of [^{14}C]CDP to [^{14}C]dCDP was measured as described in Methods. Each value is the mean of three experiments (*bars* standard errors). Enzyme activity was inhibited by 65% at 2 h in HT-29 cells. *$P < 0.05$ versus control

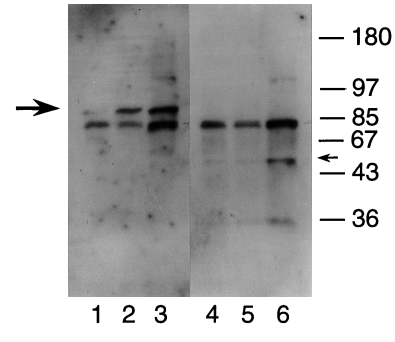


Fig. 3 Effects of HU on levels of M1 and M2. RR protein levels were assayed by immunobloting as described in Methods. M1 and M2 are indicated, respectively, by the bars at 88 kDa (*large arrow*) and 44 kDa (*small arrow*). The band at 81 kDa is unidentified and occurs reproducibly in all immunoblots. Levels of M1 (*lanes 1–3*) and M2 (*lanes 4–6*) increased in the HU-resistant variants grown in 100 μ*M* HU (*lanes 2, 5*) or 200 μ*M* HU (*lanes 3, 6*), as compared with wild-type cells (*lanes 1, 4*)

with 1000 μ*M* HU for varying times showed a decrease in RR activity, with a maximum decrease at 12–24 h and partial recovery of activity up to 48 h (Fig. 2). When wild-type cells were treated with 1000 μ*M* HU for 6, 12 or 24 h, the RR activity decreased by 40%, 65% and 48% ($P < 0.05$), respectively. The addition of IFN did not augment this effect. There was no effect of 1000 μ*M* HU on RR activity in resistant cells. IFN alone had no effect on RR activity in wild-type or R200 cells.

Effects of HU + IFN on RR protein levels in sensitive and resistant cell types

Levels of both M1 and M2 were higher in the resistant variants (Fig. 3). Cells were treated with 1000 μ*M* HU with or without 500 U/ml IFN, or with neither, for various times. Levels of M1 or M2 were quantitated by SDS-PAGE followed by immunobloting (Fig. 4). There was no consistent effect of 500 U/ml IFN or HU with or without IFN on levels of either M1 or M2 in either the sensitive HT-29 cells or the resistant variants.

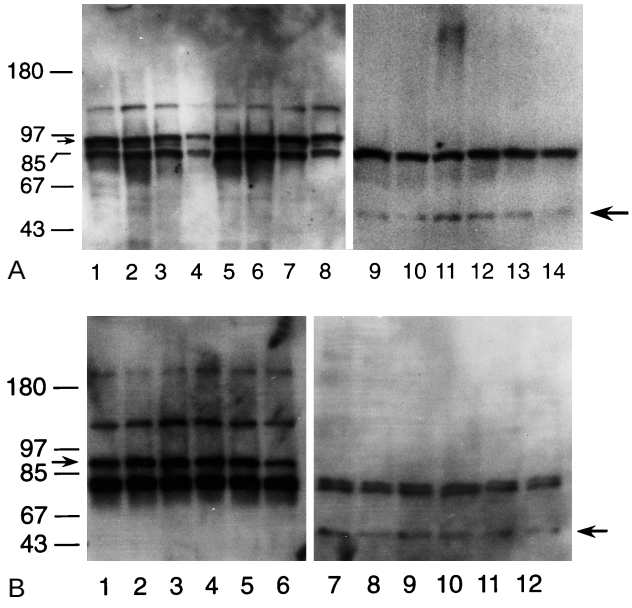


Fig. 4A, B Effects of HU with or without IFNα on M1 and M2. HT-29 (**A**) or R200 (**B**) cells were treated with 1000 μ*M* HU, 500 U/ml IFN, or the combination for 24–72 h. Protein was prepared and levels of RR subunits measured by immunoblot as described in Methods. (*arrows, left panels* M1 at 88 kD; M2, *arrows, right panels* M2 44 kD). (**A**) *lanes 1, 9*: control, *lanes 2, 10* HU × 24 h, *lanes 3, 11* HU × 48 h, *lane 4* HU × 72 h, *lanes 5, 12* IFNα, 500 U/ml IFNα, × 24 h, *lanes 6, 13* HU + IFNα × 24 h, *lanes 7, 15* HU + IFNα × 48 h, *lane 8* HU + IFNα × 72 h. (**B**) *lanes 1, 7* control, *lanes 2, 8* HU × 24 h, *lanes 3, 9* HU × 48 h, *lanes 4, 10* 500 U/ml IFNα × 24 h, *lanes 5, 11* HU + IFNα × 24 h, *lanes 6, 12* HU + IFNα × 48 h

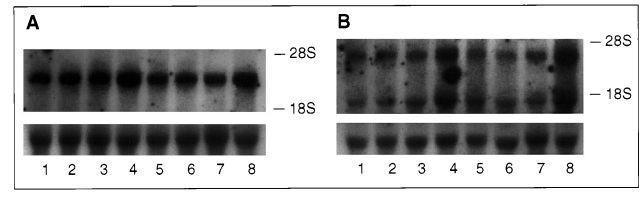


Fig. 5 Effects of HU on levels of M1 (*panel A, top*) and M2 (*panel B, top*) mRNA. HT-29 cells were treated with 1000 μ*M* HU with or without 500 U/ml IFN for 1, 4 or 24 h. Total RNA was isolated as described in Methods and analyzed by Northern blot (*Lane 1* control, *lane 2* HU × 1 h, *lane 3* HU × 4 h, *lane 4* HU × 24 h, *lane 5* IFN × 24 h, *lane 6* HU + IFN × 1 h, *lane 7* HU + IFN × 4 h, *lane 8* HU + IFN × 24 h). Two species of mRNA for M2 are apparent. Levels of mRNA for GAPDH were employed as a control (*bottom panels*)

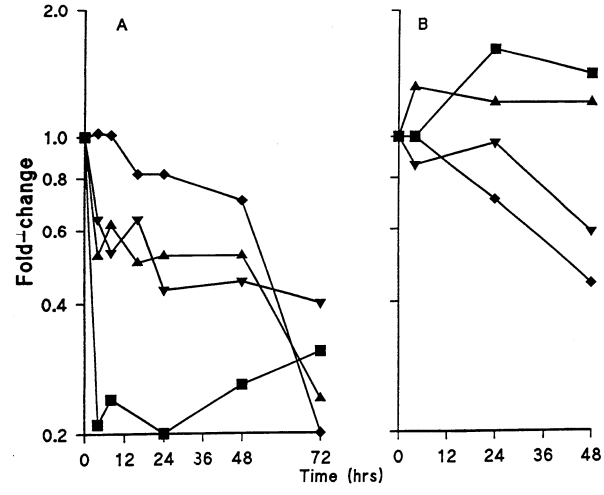


Fig. 6A, B Effects of HU on levels of dNTPs in HT-29 and R200. HT-29 cells (**A**) or R200 cells (**B**) were treated with 1000 μ*M* HU for 4–72 h. Levels of dNTPs were assayed as described in Methods. (◆ dTTP, ▼ dGTP, ▲ dCTP, ■ dATP). Levels of dATP were rapidly depleted in HT-29 cells by 80%, but not in R200 cells, within 4 h. Levels of dCTP and dGTP fell to 50–60% of baseline within 4 h in HT-29 cells, but remained stable for at least 24 h in the resistant variant. Levels of dTTP fell slowly in the HT-29 cells with comparable decreases in the HU-resistant cells. Each value is the mean of six replicates; standard errors are smaller than the point sizes. The experiment shown is representative of three performed

Effects of HU + IFN on RR mRNA in sensitive and resistant cell types

In the wild-type HT-29 cells treated with 1000 μ*M* HU there was a twofold increase in M1 mRNA at 24 h and a two- to fourfold increase in M2 mRNA at 24 h (Fig. 5). There was no effect of 500 U/ml IFN on expression of M1 or M2.

Effects of HU + IFN on pools of dNTPs in sensitive and resistant cell types

As shown in Fig. 6, treatment of HT-29 cells with 1000 μ*M* HU for 2–72 h resulted in profound depletion of pools of all dNTPs with dATP being depleted by 80% within 2 h and with dTTP being relatively unaffected up to 12 h. The most important difference between HT-29 and R200 cells was the absence in R200 cells of the decrease in pools of dNTPs at 4–12 h (Fig. 6). In contrast to the wild-type cells, pools of dNTPs did not fluctuate significantly during the first 24 h. There was a downward trend for both dGTP and dTTP at 24 h, which became significant at 48 h.

As shown in Table 1, when levels of dNTPs were measured at 24 h in both sensitive and resistant cells treated with 1000 μ*M* HU with or without 500 U/ml IFN, the addition of IFN to HU resulted in 25% depletion of pools of dGTP in the resistant cells versus no effect for HU alone. Pools of the other dNTPs were unaffected in the resistant cell line. Sensitive cells were likewise unaffected by the addition of IFN. Pools of dNTPs were measured in R200 cells at 6 h after HU treatment with or without 500 U/ml IFN. There was no effect of IFN on pools of dNTPs.

Table 1 Effects of incubation with HU with or without IFN for 24 h on pools of dNTPs. Wild type (wt) or resistant cells (R200, grown in HU, 200 μM, continuously) were incubated with 1000 μM HU with or without 500 U/ml IFN or with neither (control), for 24 h. Cells were extracted and levels of dNTPs measured by DNA polymerase assay. Results are expressed as pmol/4×10^6 cells. Each value is the mean of six replicates

dNTP	Cell type	Control	HU	HU + IFN
dGTP	wt	1.98 ± 0.02	0.21 ± 0.01	0.40 ± 0.01
	R200	1.49 ± 0.03	1.55 ± 0.02	1.12 ± 0.02*
dATP	wt	2.65 ± 0.34	0.03 ± 0.02	0.23 ± 0.03
	R200	4.45 ± 0.04	4.60 ± 0.03	4.09 ± 0.03
dTTP	wt	1.62 ± 0.02	0.83 ± 0.04	1.20 ± 0.14
	R200	2.23 ± 0.15	1.94 ± 0.02	1.65 ± 0.02
dCTP	wt	1.62 ± 0.03	0.74 ± 0.02	0.88 ± 0.02
	R200	1.39 ± 0.02	1.36 ± 0.03	1.15 ± 0.02

*$P = 0.004$ v HU

Discussion

The major finding from the cytotoxicity/proliferation assays was that duration of exposure to HU was as important to the antiproliferative effect as the drug concentration. This supports the use of HU as a prolonged infusion of at least 48 h, rather than as oral bolus therapy as has been traditionally employed. It may also explain, in part, the failure of HU to demonstrate reproducible clinical activity against solid tumors, despite the reproducible activity against the target enzyme, RR, in experimental solid tumor models. The availability of the parenteral formulation of HU has led to the need for the re-evaluation of the role of this agent as a cytotoxic agent or a modulator of other cytotoxic agents employed as a prolonged infusion.

The role of HU resistance was investigated employing a cell line with low-level resistance, which is likely to mimic the clinical situation. As compared with untreated wild-type cells, there was only a two- to threefold increase in levels of both subunits of RR. This was sufficient, however, to completely abrogate inhibition of enzyme activity for up to 72 h and to almost completely abrogate perturbation of dNTP pools for up to 24 h at concentrations of HU that are clinically achievable by infusional therapy. While clinical resistance is likely to be multifactorial and to result from multiple compensatory mechanisms at the cellular level, we have demonstrated in our tumor model system that even small increments in levels of M1 and M2 may be sufficient for solid tumors to compensate for inhibition of remaining enzyme, thus confirming one potential mechanism likely to be observed in vivo.

The time course for the biochemical effects of HU was rapid. HU (1000 μM), inhibited RR activity within 1 h, and at 12–24 h by over 67%. This correlated with a rapid fall in levels of dATP, dCTP and dGTP by 4 h. Induction of M1 and M2 occurred more slowly. Levels of mRNA for both M1 and M2 increased following 24 h of treatment with HU with or without IFN, but not at 1 or 4 h. Beyond 24 h there was partial recovery of enzyme activity. Despite fluctuations in levels of dNTP pools, enzyme activity and enzyme expression, levels of M1 and M2 were relatively constant at 24 and 48 h, whether the cells were treated with HU alone, IFN alone or the combination. Thus, the wild-type human colon cancer cells were able to compensate for prolonged, intensive exposure to HU with rapid induction of enzyme sufficient to compensate for the induction of perturbations in endogenous dNTP pools at least up to 48 h. After 48 h, wild-type cells were profoundly depleted of all four dNTPs, an effect correlating well with the changes observed on flow cytometry indicating accumulation of cells in S-phase, where presumably perturbations in pools of dNTPs result in the inhibition of DNA synthesis. In the resistant variants, levels of M1 and M2 were increased, levels of enzyme activity were increased, and the cells were immune to perturbations in dNTP pools, at least for the first 24 h. After 24 h, there was evidence of depletion of dCTP and dGTP, and this correlated with the inability of cells to survive at higher concentrations of HU than the 200 μM in which they were grown.

The activity of IFN was investigated in combination with HU for several reasons. First, IFN has been shown to effectively modulate the activity of another antimetabolite, 5FU [20]. Second, as the regimen of 5FU + IFN has been effective in the treatment of colorectal cancer and as HU has been shown to modulate the activity of 5FU in vitro, we had planned to incorporate HU as a parenteral infusion into the 5FU + IFN regimen. Thus, it was important to determine whether HU + IFN interacted independently of 5FU.

It was of interest, then, that IFN was capable of augmenting the cytotoxicity of HU in a dose-dependent fashion against the wild-type colon cancer cell lines. Furthermore, this occurred at concentrations of both agents and using schedules of both agents that are clinically achievable. It was disappointing that a mechanism of enhancement of cytotoxicity by IFN could not be identified. There was no effect on expression of M1 or M2 or on RR activity. There was a very modest inhibition of levels of dGTP at 24 h which is unlikely to account for the augmented cytotoxicity. Furthermore, IFN at these concentrations was unable to reverse resistance to HU in the R200 cells, and therefore it was not surprising that IFN failed to affect expression of M1 or M2, levels of RR, or dNTP pools in these cell lines.

In conclusion, our results support the use of HU as a prolonged, parenteral infusion, rather than more frequent oral administration, since we demonstrated that the inhibitory effects on RR and dNTPs continue for at

least 24 h and on cell proliferation for up to 72 h after HU treatment. Both HU-resistant cells and wild-type cells exposed to HU for 24 h expressed higher levels of the M1 and M2 subunits; levels of mRNA for both M1 and M2 increased by 24 h, but not by 4 h. IFN augmented the antiproliferative effects of HU, but these effects did not appear to result from independent cytokinetic effects of IFN nor from augmentation of the effects of HU by IFN against RR.

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