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Effects of Perturbations of Pools of Deoxyribonucleoside Triphosphates on Expression of Ribonucleotide Reductase, a G_1/S Transition State Enzyme, in p53-Mutated Cells

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ABSTRACT. Effects of drug treatment with antimetabolites on a human colon cancer cell line, SW480, were studied. Cells were treated with 10 μM of 5-fluorouracil (5FU), an inhibitor of pyrimidine synthesis, or 1000 μM of hydroxyurea (HU), an inhibitor of both purine and pyrimidine syntheses, or the combination. Recombinant α-2a-interferon (IFN), 500 U/mL, also was employed, as this augments the effects of both antimetabolites *in vitro* and *in vivo*. The predominant effect of this combination was to block cells in early S phase as measured by 5-bromo-2′-deoxyuridine (BrdUrd) incorporation. By 24 hr, 86% of the cells had accumulated in S phase, but failed to progress to G_2/M. This was accompanied by an early, rapid decline in all four deoxyribonucleoside triphosphates (dNTPs) by 38–86% at 4–24 hr. Despite these effects, expression of the G_1/S transition state enzyme, ribonucleotide reductase (RR), increased at 24 hr as measured by a 3 to 5-fold increase in mRNA levels for the M2 subunit, in the absence of a measurable effect on protein levels. The rise in levels of RR mRNA and the continued progression of cells into S phase were associated with a synergistic inhibition of cell cycle proliferation resulting from treatment with the three-drug combination. This suggests that in the presence of antimetabolite-induced depletion of dNTPs, SW480 cells, which lack a normal *p53* gene, will proceed into S phase, and that this is associated with a rise in expression of the G_1/S transition state enzyme, RR. Cells arrested in S phase by a p53-independent mechanism will undergo a synergistic enhancement of cell death. BIOCHEM PHARMACOL **55**;9:1353–1360, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ribonucleotide reductase; deoxyribonucleoside triphosphates; 5-fluorouracil; hydroxyurea; *p53*; cytokinesis

In normal cells, the response to DNA damage is governed by the state of differentiation of the cell [1] and the status of specific growth-regulating genes, such as *p53* and *Rb* [2]. In cells that have undergone malignant transformation, the situation is more complex. While structural DNA damage may result in G_1 arrest [3], not all malignant cells sustaining DNA damage will undergo a p53-mediated G_1 arrest [4], cells with mutant or absent p53 may lose the ability to arrest in G_1 [5], and γ-radiation-induced apoptosis can occur in the absence of p53 [6].

Metabolic perturbations in the absence of DNA damage can also result in alterations in cell-regulatory pathways, but these have been less well studied than the effects of DNA damage. Furthermore, these effects may differ from those resulting from treatment with DNA-damaging agents. In normal diploid fibroblasts, treatment with agents that depleted nucleotide pools resulted in activation of a DNA damage-independent arrest mechanism mediated by p53 [7]. In contrast to effects induced by structural DNA damage, the resultant quiescent state was reversible and was not associated with morphologic changes or apoptosis. Furthermore, treatment with antimetabolites, such as PALA†,[1] which induced depletion of nucleotide pools in this model, also arrested cells in G_1 despite the induction of a variety of cell cycle pro-regulatory genes, such as cyclin D1 and cyclin E. The effects of nucleotide depletion on cell cycle regulatory genes likely vary between specific cell types. For example, while SW620 and HT29 human colon carcinoma cells both lack functional p53, treatment with the fluoropyrimidine FdUrd resulted in a block at the G_1/S boundary in SW620 cells, suggesting the presence of a *p53*-independent checkpoint, whereas HT29 cells progressed into S with a concomitant increase in cell death [8]. Therefore, possible responses to antimetabolites that per-

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† *Abbreviations:* PALA, *N*-(phosphonacetyl)-L-aspartate; BrdUrd, 5-bromo-2′-deoxyuridine; HU, hydroxyurea; 5FU, 5-fluorouracil; dNTP, deoxyribonucleoside triphosphate; IFN, recombinant α-2a-interferon; FITC, fluorescein isothiocyanate; FdUrd, fluorodeoxyuridine; RR, ribonucleotide reductase; and SRB, sulforhodamine B.

turb nucleotide pools may include p53-mediated or p53-independent G_1 growth arrest or, alternatively, activation of E2F-responsive G_1/S transition enzymes with resultant progression into S. Once in S phase, alterations in the ratios of the various nucleotide pools can result in the accumulation of DNA damage, leading to apoptosis or non-apoptotic cell death (sensitive cells), or, alternatively, cells may survive this metabolic insult through prevention or repair of DNA damage (resistant cells).

For any specific cell type, it is problematic to attempt to predict *a priori* how metabolic perturbations would affect cell growth and survival. Specifically, there is no single biological determinant of cellular resistance or sensitivity. The ability of cancer cells to survive disruption of pools of dNTPs by antimetabolites is likely a complex process, which may depend, in part, on the *pattern* of disruption (which pools of dNTPs either increase or decrease, and their ratios) and the *magnitude* of the disruption of these dNTP pools. We have previously studied [9] this phenomenon in SW480 cells, focussing on the depletion of TTP. We now chose to study the effects of simultaneous depletion of both pyrimidine and purine pools. SW480 cells are of interest because they have a single mutant *p53* allele, lack functional p53 activity [10], and do not undergo p53-mediated G_1 arrest following γ-radiation [11]. This is highly relevant to the clinical situation because missense mutations of *p53* are the most common genetic mutations in human malignancies [12, 13], and the specific mutation in SW480 cells is the second most common *p53* missense mutation in human cancers [14, 15]. Prior studies [9] using single antimetabolites that induced modest perturbation of pools of dNTPs failed to result in significant inhibition of cytokinesis. We hypothesized that increased disruption of both pyrimidine and purine pools in cells lacking p53 might trigger an alternative mechanism, which might either inhibit progression into S or prevent induction of specific G_1/S transition state enzymes, thereby protecting cells from misincorporation of dNTPs, resulting in accumulation of DNA damage.

To deplete the endogenous pyrimidine nucleotide TTP, SW480 cells were treated with 5FU a fluorinated pyrimidine that is anabolized to the fluorinated nucleotide FdUMP, which, in turn, covalently binds thymidylate synthase (TS), resulting in depletion of thymidine nucleotide precursors for DNA synthesis. To simultaneously deplete purine nucleotide pools, cells were treated with HU, which inhibits the tyrosine free radical on M2, the small subunit of RR [16, 17]. IFN was also employed, because it augments the activity of both 5FU [18] and HU [19]. Effects of the drug combination on markers for cell growth and survival were correlated with the effects of the drugs on perturbation of nucleotide pools.

MATERIALS AND METHODS

Cell Culture

SW480 cells were maintained in RPMI 1640 (Life Technologies) with 10% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin–streptomycin at 37° in 5% CO_2. Cells were removed from standard culture medium 24 hr prior to drug exposure and incubated in folate-free RPMI 1640 (Life Technologies) with 10% dialyzed FBS (D-FBS) and 80 nM 5-methyltetrahydrofolic acid (Sigma) without antibiotics. The doubling times of cells were unaffected by the latter medium.

Drugs and Reagents

Recombinant IFN was a gift of Hoffmann-LaRoche, and hydroxyurea was a gift of Dr. Terry Dugan, Bristol-Myers-Squibb. DNA polymerase I, large fragment (Klenow fragment), and Dulbecco's phosphate-buffered saline (D-PBS) were from Life Technologies. Oligonucleotides were from American Synthesis and also from the Oligonucleotide Facility at the Albert Einstein Cancer Center. All other reagents were from the Sigma Chemical Co.

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. [2,8-^{3}H]Deoxyadenosine triphosphate, 32 Ci/mmol, and cytidine 5′-diphosphate, [U-^{14}C], 400 μCi/mmol, were from Moravek Biochemicals.

Preparation of Cell Extracts for dNTP Measurements

Cells were incubated in folate-free RPMI 1640 with 10% D-FBS and 80 nM of 5-methyltetrahydrofolic acid with or without 5FU, HU, and/or IFN for various time intervals in 6-well Falcon tissue culture plates (Becton Dickinson). Without washing, medium was poured off rapidly, and 200 μL of ice-cold perchloric acid (PCA) was added for 30 min. The extract was centrifuged for 2 min at 12,000 *g*, and then the supernatant was neutralized to pH 7.4 with 400 μL of alamine:Freon, as previously described [19]. Extracts were again centrifuged for 2 min, and the top layer was removed for the assay.

Measurement of dNTP pools

To assess the effects of 5FU on RR activity *in situ*, pools of dNTPs were measured following treatment with HU, 5FU + IFN, or 5FU + IFN + HU. The DNA polymerase assay, modified from that of Sherman and Fyfe [20], was employed as previously described [21]. 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 $\geq$ 0.99 were obtained routinely.

Flow Cytometry

Cells were plated 24 hr before drug treatment. Cells were incubated in folate-free RPMI 1640 with 10% D-FBS and

80 nM of 5-methyltetrahydrofolic acid with or without 5FU, HU, and/or IFN for various time intervals in 25-cm^2 Falcon tissue culture flasks (Becton Dickinson). Following drug exposure, cells were labeled for 1 hr with 20 μM of BrdUrd in drug-free medium as described [7]. Cells were harvested by trypsinization, fixed in 70% ethanol at 4°, washed with PBS containing 0.5% BSA, and centrifuged as described [22]. The pellet was suspended in 0.1 M of HCl containing 0.5% Triton X-100 in water to extract histones. After a 10-min incubation on ice, cells were washed with water, then boiled, and cooled rapidly to denature the DNA. Next, cells were incubated with 0.5 μg of anti-BrdUrd-FITC (Pharmingen) dissolved in PBS containing 0.1% BSA for 30 min. After washing with PBS, cells were resuspended in 300 μL of PBS, and then 400 μL of propidium iodide (PI) buffer (3.4 mM of sodium citrate, 0.5 mM of Tris-HCl, 0.1% NP-40, 3 mM of spermine tetrahydrochloride, and PI, 50 μg/mL). After a 30-min incubation, samples were analyzed on a Becton–Dickinson FACScan Flow Cytometer for DNA content (PI fluorescence) and BrdUrd incorporation (FITC fluorescence).

Measurement of RR Subunits M1 and M2 by Immunoblot

Cells were exposed to drug for various time points as described above. Protein was isolated, and the assay was performed as described previously [19]. Blots were incubated with 1 μg of antibody to M1 (AD203, InRo Biomedtek) [23] or 2 μg of antibody to M2 (the monoclonal rat antitubulin antibody, YL1/2, Accurate Chemical) [24], for 3 hr at 4°. Blots were incubated with 2 μg of the appropriate biotinylated second antibody, and then with streptavidin-alkaline phosphatase (Life Technologies), and protein was detected by the addition of Lumi Phos (Life Technologies), 1–2 mL/blot, followed by exposure to Kodak X-omat film for 3–5 hr at room temperature. The authenticity of the bands was confirmed by comparison to recombinant mouse R1 ribonucleotide reductase subunit (supplied by Dr. Lars Thelander, University of Umea), which shares 97% homology with the human subunit.

Measurement of RR Subunits M1 and M2 RNA by Northern Analysis

Cells were treated without or with 5FU, HU, IFN, or combinations of these agents for various durations. RNA was isolated either in guanidine isothiocyanate (GIT) buffer layered over a cesium chloride cushion or in a single-step procedure employing a mono-phasic solution of phenol and GIT (TRIzol Reagent, Life Technologies), which gave equivalent results. Northern analysis was performed on an agarose-formaldehyde gel. Blots were transferred to nylon membranes and probed with ^{32}P-labeled probes specific for either M1 or M2 [25].

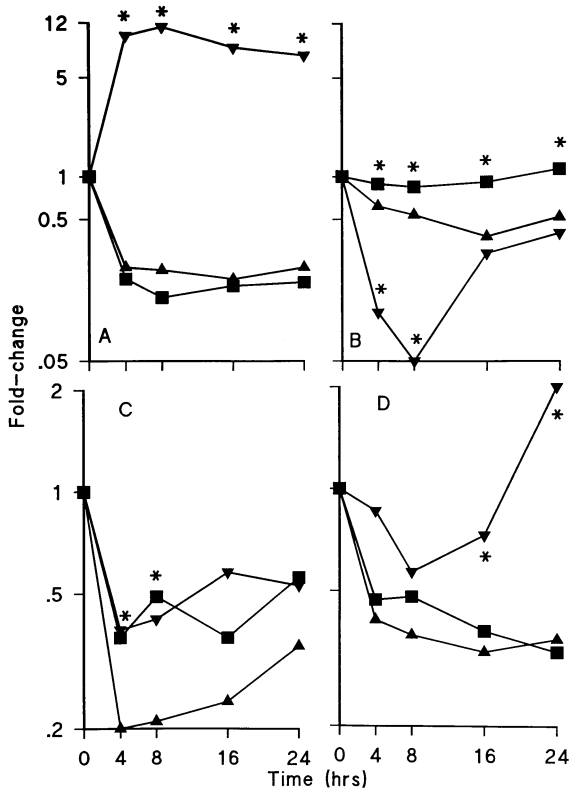

FIG. 1. Effects of treatment on pools of dNTPs. Cells were treated with HU (1000 μM) alone (■), 5FU (10 μM) + IFN (500 U/mL) (▼), or 5FU, HU, and IFN (▲), for 0–24 hr. dNTPs were measured as described in Materials and Methods at selected time points. (A) dATP, (B) TTP, (C) dGTP, and (D) dCTP. Points: mean of 3 experiments performed in replicates of 6. Standard error bars are within the points. *$P < 0.01$ vs 5FU + HU + IFN.

Measurement of Cell Growth

Cell proliferation was estimated using a slight modification of the SRB assay [26]. Results obtained with this assay were similar to those obtained with a clonogenic assay. Cells were incubated with drug for various time points. Preliminary tests demonstrated no significant loss of cells at 24 hr, and >95% of the cells were viable by trypan blue exclusion after removal of drug. Drug-containing medium was removed and replaced with fresh medium. Cells were incubated in the absence of drug for 7 days and then were fixed and stained exactly as described. Correlation coefficients for standard curves comparing number of cells and absorbance were routinely ≥ 0.99.

RESULTS

Effects of Drug Treatment on Nucleotide Pools

The effects of HU, 5FU + IFN, and the three-drug combination on pools of dNTPs were very different. As shown in Fig. 1, treatment with HU (1000 μM) alone resulted in depletion of pools of dATP, dGTP, and dCTP by 52–86% within 8 hr. For dATP and dCTP, this effect persisted for 24 hr. For dGTP, there was a partial rebound

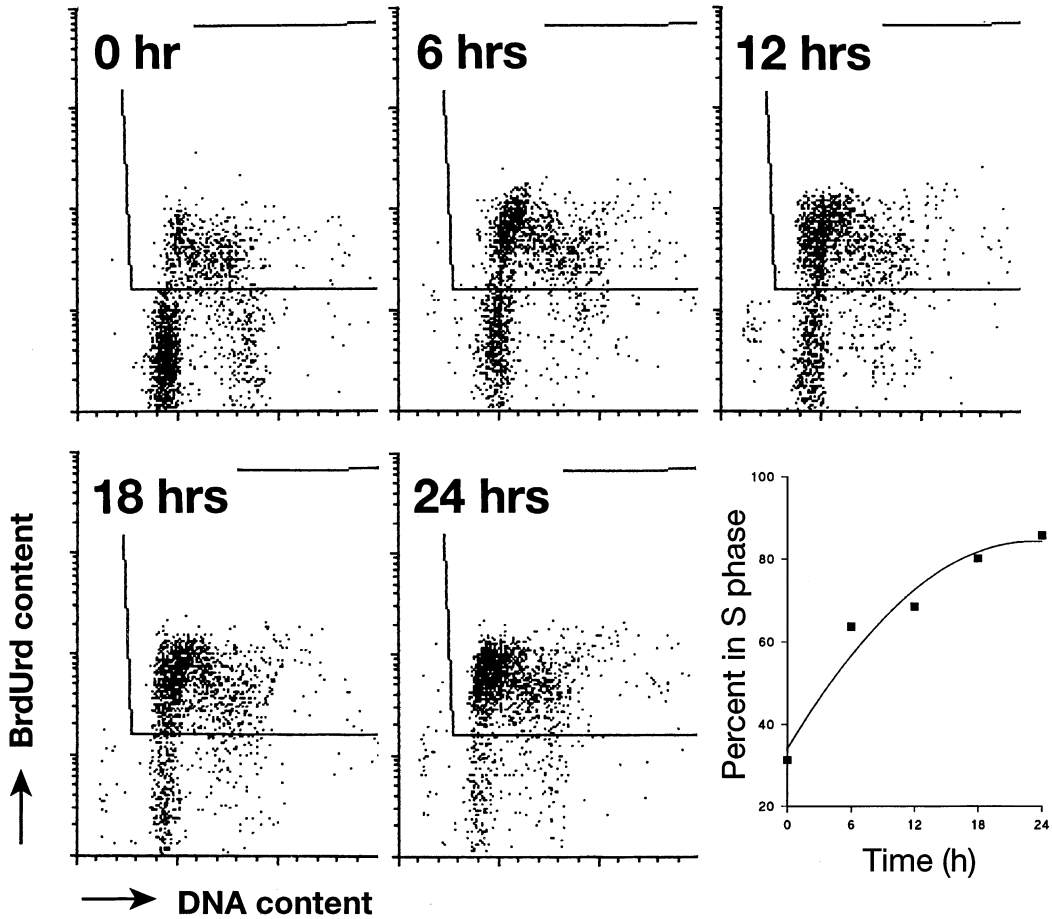

FIG. 2. Effects of drug treatment on cell cycle progression. Cells were treated with 5FU (10 μM), HU (1000 μM), and IFN (500 U/mL), for 0–24 hr. Cells were labeled with 20 μM of BrdUrd for the last 60 min of drug treatment. They were then prepared for two-parameter flow cytometry as described in Materials and Methods. As shown in sequential panels, cells continued to progress into S phase, as indicated by points above the line, at a declining rate, but failed to proceed further into G_2/M. The graph indicates the percent of cells in S phase as indicated by R2 gating at specific time points. Results shown are representative of 3 experiments performed in duplicate, which demonstrated identical results.

at 8–24 hr from 63% depletion to 44% depletion. Treatment with HU alone resulted in only a 17–19% depletion of pools of TTP at 4–8 hr, followed by an 11–22% increase in TTP levels above baseline levels at 16–24 hr.

In contrast to the effects of the single agent HU on dNTP levels, treatment with the combination of 5FU (10 μM) + IFN (500 U/mL) resulted in rapid and early depletion of TTP levels by 95%, with a rapid rise in pools of dATP by >10-fold, both within 4 hr. The effects on pools of dCTP and dGTP were not as dramatic, with a 40–75% decrease within 4–8 hr followed by a small (dGTP) or large (dCTP) rebound at 8–24 hr.

The effects of the combination of 5FU (10 μM) + IFN (500 U/mL) + HU (1000 μM) on pools of dATP and dCTP were similar to those of HU alone, and differed markedly from the effects of 5FU + IFN (Fig. 1). Effects on levels of dGTP and TTP were different from both HU and 5FU + IFN, however. In the case of TTP, the effects of the three-drug combination were intermediate between those of HU and 5FU + IFN, and different from both ($P < 0.01$). Treatment with this combination did result in a sustained inhibition of TTP by about 38–62% from 4–24 hr, although the presence of HU abrogated the earlier inhibitory effects of 5FU + IFN. Furthermore, the three-drug combination also resulted in greater depletion of dGTP ($P < 0.01$) than either 5FU + IFN or HU alone at early time points, 4–8 hr, which partially rebounded at 16–24 hr.

Effects of Drug Treatment on Cell Cycle Progression

As shown in Fig. 2, SW480 cells treated with the combination of 5FU (10 μM), HU (1000 μM), and IFN (500 U/mL) continued to progress into S phase from 0–24 hr, although at a declining rate, as measured by incorporation of BrdUrd. At 24 hr, the percentage of cells in S phase had increased from 31 to 86% of treated cells. Cells failed to progress beyond early S phase, resulting in depletion of both the G_1 and G_2/M compartments.

When either HU or 5FU + IFN were studied individually at the same concentrations, the effects on cell cycle progression were nearly identical to that of the three-drug

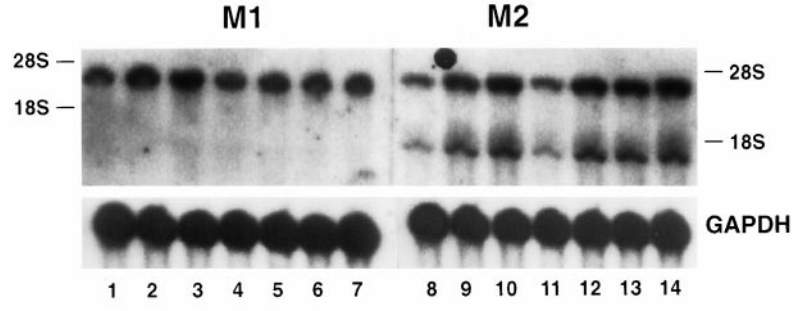

FIG. 3. Effects of treatment on M1 and M2 mRNA. Cells were treated without (lanes 1, 8) or with either 10 μM of 5FU (lanes 2, 9), 1000 μM of HU (lanes 3, 10), 500 U/mL of IFN (lanes 4, 11), 5FU + IFN (lanes 5, 12), 5FU + HU (lanes 6, 13), or 5FU, HU, + IFN (lanes 7, 14) for 24 hr. mRNA was prepared and analyzed as described in Materials and Methods. There was a < 2-fold increase in M1 and a 3 to 5-fold increase in both species of M2 with drug treatment, but not IFN treatment, as measured by densitometry. GAPDH was employed as a control. Autoradiographs are representative of 3 experiments with identical results.

combination (data not shown), despite the profound differences noted on pools of dNTPs.

Effects of Drug Treatment on Expression of RR Subunits M1 and M2

The allosteric effector subunit of RR, M1, is constitutively expressed, while the smaller M2 subunit, containing the non-heme iron is only expressed during the transition from G_0 or G_1 to S phase [10, 27, 28]. To assess the effects of drug treatment on expression of RR, Northern analysis was performed on extracts of cell samples treated with 5FU (10 μM), HU (1000 μM), with or without IFN (500 U/mL) for 24 hr. As shown in Fig. 3, treatment with either 5FU alone, HU alone, or the combination of 5FU and HU, with or without IFN resulted in a 3 to 5-fold increase in levels of both subspecies of M2 mRNA, effects consistent with progression of cells into S phase observed by flow cytometry.

The effects on levels of M1 mRNA were different. Treatment with either 5FU or HU resulted in small increases in M1 mRNA; however, these were consistently < 2-fold. IFN treatment alone had no effect on expression of either M1 or M2.

As shown in Fig. 4, treatment of SW480 cells with either 5FU, HU, or IFN for 24 hr did not result in any changes in the levels of M1 or M2 protein, despite the increases in mRNA observed above.

Effects of Drug Treatment on Cell Proliferation

As shown in Fig. 5, IFN augmented the cytotoxicity of both 5FU and HU against SW480 cells at clinically achievable concentrations. To determine whether there was an interactive effect of the drugs, the effects of the combination on cell proliferation were analyzed by median effects analysis [29]. The EC_{50}, EC_{70}, and EC_{90} values for 5FU in combination with a fixed concentration of IFN, 500 U/mL, were 1.1, 2.3, and 7.7 μM, respectively; for HU alone, they were 129, 244, and 677 μM, respectively; and for HU in combination with 5FU (0.25 to 8.0 μM) + IFN (500 U/mL), they were 117, 146, and 209 μM, respectively. Synergy for each of the doublets has already been demonstrated [18, 19], and the effects of the three-drug combination were also synergistic.

DISCUSSION

In contrast to our working hypothesis, rapid depletion of both purine and pyrimidine pools, induced by treatment with 5FU, HU, and IFN, was not associated with a G_1 arrested state in the SW480 tumor model system employed

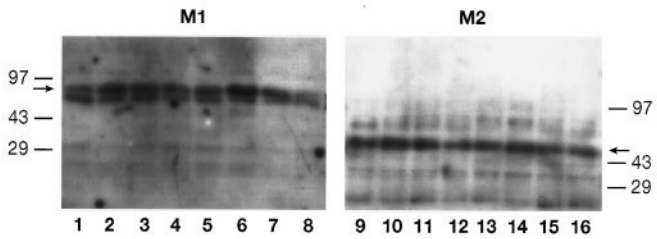

FIG. 4. Effects of drug treatment on M1 and M2 protein. Cells were treated without (lanes 1, 9) or with either 10 μM of 5FU (lanes 2, 10), 1000 μM of HU (lanes 3, 11), 500 U/mL of IFN (lanes 4, 12), 5FU + IFN (lanes 5, 13), 5FU + HU (lanes 6, 14), HU + IFN (lanes 7, 15), or 5FU, HU, + IFN (lanes 8, 16) for 24 hr. Protein was prepared and analyzed as described in Materials and Methods. There was no change in intensity of either the 88-kDa M1 subunit or the 44-kDa M2 subunit as measured by densitometry with any treatment. The band at 81 kDa was unidentified and occurred reproducibly in all immunoblots. This autoradiograph is representative of 3 experiments showing identical results.

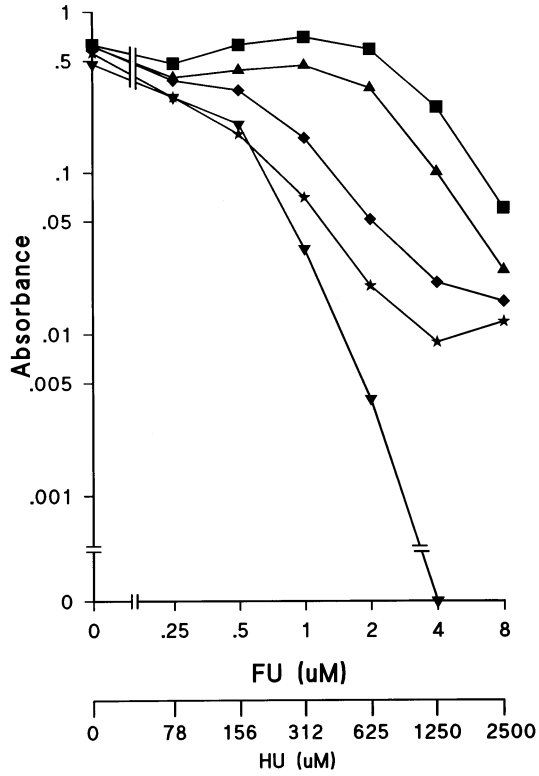

FIG. 5. Effects of drug treatment on cell proliferation. Cells were treated with 10 μM of 5FU (■), 5FU + 500 U/mL of IFN (▲), 1000 μM of HU (◆), HU + 500 U/mL of IFN (★), or 5FU + HU + IFN (▼) for 24 hr. Effects on cell proliferation were measured by the SRB assay as described in "Materials and Methods". Points: means of 3 experiments performed in replicates of 6; standard error bars are within the symbols.

in these studies. In the absence of a functional *p53* gene, cells treated with the three-drug combination continued to progress into S phase, eventually accumulating 86% of the cell population in S. This was associated with increased expression of the G_1/S transition state enzyme, RR, particularly the inducible subunit, M2, despite the marked perturbations in pools of dNTPs.

In *p53*-intact cells, effects on perturbations of nucleotide pools may be associated with p53-induced apoptosis [7, 30]; however, mutations in *p53* have been reported to result in resistance to cytotoxic drugs [30, 31], which may, in some cases, be related to induction of *mdr*1 [32], and also, presumably, to a failure to undergo apoptosis. In contrast, in our tumor model system, failure to arrest at the G_1/S boundary in the presence of significant perturbations of nucleotide pools was associated with a significant augmentation in cell death. Thus, the effects of dNTP perturbation appear to differ from the effects of DNA-damaging agents, in that progression into S phase, in the absence of p53-mediated apoptosis, is accompanied by an increase in cell lethality, as measured by decreased cell proliferation. Furthermore, this is consistent with effects of the quinazoline-based antifolate derivative ZD1694, in which cell death was also a function of progression into S phase with subsequent accumulation of DNA damage [33].

In contrast to the effects on cell cycle progression where 5FU + IFN, HU alone, or the three-drug combination all caused essentially identical accumulation of cells in S phase, the effects on cell proliferation were quite different. Using a rigorous analysis of synergy, there was a positive interaction between the agents. This, together with the studies demonstrating very different patterns of nucleotide pool perturbation, suggests that it was likely that the magnitude of the disruption of the nucleotide pools, rather than the cytokinetic effects, was the major determinant of cell death. Depletion of specific nucleotide pools can result in misincorporation of dNTPS, point mutations, and DNA double-strand breaks. Whether the critical factor is depletion of dNTPs or the creation of imbalances in pools of dNTPs is unclear. Imbalances in pools of dNTPs in murine FM3A cells resulted in activation of a cellular endonuclease activity that produced DNA double-strand breaks [34]. Such an endonuclease activity was isolated from cells treated with 5FU, a fluoropyrimidine. In this complex scheme, depletion of TTP resulted in incorporation of dUTP into DNA, with subsequent excision by DNA-uracil-*N*-glycosylase causing single-strand breaks [35]. However, these were not lethal events *per se*, but were associated with subsequent augmentation in the ratio of pools of dATP to dGTP, mediated by effects on ribonucleotide reductase, which resulted in activation of an endogenous endonuclease, which, in turn, produced lethal DNA double-strand breaks [36]. Furthermore, while dATP pools were depleted significantly at 4 hr in our tumor model system, the dATP/dGTP ratio still increased markedly as dGTP pools fell by 80%. Nevertheless, the patterns of dNTP pool changes observed in our system differed markedly from those observed in FM3A cells, suggesting that events other than the dATP/dGTP ratio are critical for the increase in cell death observed with the combination of 5FU + HU + IFN. Furthermore, it is likely that it is the magnitude of the disruption of all four dNTPs that results in cell death, rather than a specific change in the size of two dNTP pools. This is consistent with our previous finding that more thorough depletion of dTTP results in a significant increase in DNA double-strand breaks [9].

While intriguing, this hypothesis leaves many unanswered questions. These include the specific dNTPs that must be perturbed to induce endonuclease activity, the magnitude in the changes of the pools and pool ratios that are required to optimize this effect, the cell specificity of such effects, and the optimal timing of such events. Recent data also suggest the importance of compartmentalization and channeling of pools of dNTPs [37]. Finally, the discordance between the synergistic effects observed on cell proliferation with the three-drug combination and the absence of a synergistic effect on cytokinesis, and specifically accumulation of cells in S phase, suggests that it is likely that the perturbation of dNTP pools, rather than cytokinetic effects, is most closely associated with increased cell death.

The effects of treatment on expression of the M2, and to a lesser extent M1, subunits of RR were complex. Levels of

M2 mRNA rose 3 to 5-fold following treatment with 5FU or HU or the combination, consistent with enhanced expression during the transition from G_1 to S phase. Thus, with 86% of the cell population accumulating in S, a rise in M2 confirms that, despite the perturbations in nucleotide DNA precursors, cells continue not only to incorporate BrdUrd but also to increase levels of the RR subunit necessary for synthesis of additional dNTPs.

Despite the increase in levels of mRNA for both M1 and M2 following treatment with either 5FU or HU, alone or in combination, there was no concomitant change in protein levels as measured by Western analysis. The disparity between effects on mRNA and protein levels may be explained by the observation that post-transcriptional regulation is critical in determining levels of M1 and M2, and has been shown to be affected by exogenous agents, such as the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate), via specific *cis*-acting elements in the 3′ untranslated region of the M1 and M2 mRNAs [38–40]. Thus, it is possible that treatment with the antimetabolites employed in this study resulted in an increase in mRNA stability, which could account for elevated mRNA levels, in the absence of an increase of protein levels.

Furthermore, the absence of a change in protein levels was associated with a decrease in RR activity as measured by a 52–86% decline in levels of dATP, dCTP, and dGTP. It is still unclear why stable protein levels should be associated with a decrease in nucleotide pools; however, we can postulate that allosteric regulation of RR (reviewed in Ref. 41) may account for such an effect.

In conclusion, we have employed a *p53* mutated tumor model system to examine the effects of combined purine and pyrimidine effects on the G_1/S transition state enzyme, RR, and the subsequent effects on nucleotide pools. In this system, antimetabolite-induced perturbations of dNTP pools failed to result in G_1 arrest, but did result in a p53-independent arrest in early S phase. Despite induction of RR, purine pools subsequently fell, and the combined depletion of both purine and pyrimidine nucleotide precursors was associated with an increase in cell lethality. Future questions remain as to how these effects are linked to expression of the various cell cycle regulatory genes, and to how these effects can be made selective for cells that have undergone malignant transformation.

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