The Interaction of Hydroxyurea and Iododeoxyuridine on the Radiosensitivity of Human Bladder Cancer Cells

Mei-Ling Kuo, Keith A. Kunugi, Mary J. Lindstrom, and Timothy J. Kinsella

Departments of Human Oncology [M-L. K., K. A. K., T. J. K.] and Biostatistics [M. J. L.], University of Wisconsin Medical School, Madison, Wisconsin 53792

ABSTRACT

Biochemical modulation of iododeoxyuridine (IdUrd) incorporation into the DNA of tumor cells is a potential clinical strategy to enhance radiosensitivity and to simultaneously differentiate the sensitivity of rapidly proliferating tumor cells and more slowly proliferating adjacent normal tissues to radiation. The interactions of hydroxouracil (HU) and IdUrd were studied in a human bladder cancer cell line, 647V. Exposure of exponentially growing 647V cells to HU concentrations of 10–100 μM for one cell population doubling (24 h) resulted in no cytotoxicity as assessed by clonogenic survival. Flow cytometric analysis showed a significant increase in an early S-phase population after a 12-h exposure but a return to a normal cell cycle distribution after a 24-h exposure to 100 μM HU. Incorporation of IdUrd into DNA was increased 2-fold by coincubation with HU (100 μM) and a clinically achievable concentration of IdUrd (2 μM) for 24 h. To elucidate the mechanism of modulation, IdUTP pools were compared in 647V cells treated with 2 μM IdUrd with or without 100 μM HU. A 2-fold increase in IdUTP pools was evident within 2 h when this drug combination was used. With the use of multivariate statistical analysis, the radiosensitivity of 647V cells was compared after a 24-h exposure to various concentrations of IdUrd (0 and 2 μM) and HU (0, 10, and 100 μM). A 24-h exposure to 100 μM HU alone or to 2 μM IdUrd alone before irradiation resulted in significant (P < 0.02) radiosensitization with sensitization enhancement ratios of 1.15 and 1.27, respectively. A 24-h exposure to 100 μM HU + 2 μM IdUrd resulted in even more significant (P = 0.001) radiosensitization, which was found to be greater than additive response (sensitizer enhancement ratio, 1.76 observed compared with 1.37 expected). No radiosensitization was found with a 12-h exposure to 100 μM HU alone. The mechanism of biochemical modulation of IdUrd by hydroxyurea has been proposed as increasing the IdUTP pools by stimulating enzymes in the thymidine salvage pathway and subsequently enhancing IdUrd incorporation and radiosensitization.

INTRODUCTION

IdUrd, an analogue of thymidine, is a known radiosensitizer when incorporated into DNA (1–3). Initially, IdUrd competes with thymidine for conversion to its monophosphate form (IdUMP) by thymidine kinase (Ref. 4; Fig. 1). Additional phosphorylation generates IdUTP, which is used in DNA synthesis (1). Normally, cells produce dTMP through the de novo pathway by methylation of dUMP with the use of thymidylate synthase. dUMP can be produced by deamination of dCMP or by dephosphorylation of dUTP by dUTPase. dUTP is normally derived via dUDP or dCTP. Both dCTP and dCMP are derived from dCDP. dUDP and dCDP are converted from UDP and CDP by RR through the de novo synthesis pathway (Fig. 1).

RR is the rate-limiting enzyme in the de novo synthesis of deoxynucleotides. The small subunit of RR, R2, contains a tyrosyl free radical that is essential for holoenzyme activity. HU is known to inhibit RR by scavenging the free radical in the R2 subunit. The inhibition of ribonucleotide reductase activity by HU causes a decrease of deoxynucleoside triphosphate pools; for example, HU exposure resulted in a significant decrease in dATP pools in 3T6 cells (5). dCTP and dGTP pools were decreased as well, whereas dTTP pools increased. Because the de novo synthesis of dTMP is inhibited by HU, the feedback inhibition of thymidine kinase by dTTP will be reduced, thereby increasing the salvage pathway contribution of dTTP synthesis. It has been demonstrated that the increase of dTTP after exposure to HU is caused by the influx of uridine, followed by phosphorylation by thymidine kinase in cells (5).

A practical limitation to the use of IdUrd as a clinical radiosensitizer is the reported low percentage of IdUrd-DNA incorporation in some human tumors (6, 7). Because the radiosensitizing effect of IdUrd is highly associated with the amount of IdUrd incorporated into cellular DNA (2, 3, 8–12), modulation of IdUrd incorporation into DNA could further enhance the radiosensitivity of tumor cells. However, simply increasing the dose of IdUrd to theoretically achieve a higher incorporation has resulted in unacceptably severe normal tissue toxicities (13, 14). Biochemical modulation of IdUrd incorporation has been tested experimentally with the use of several drugs that modify thymidylate synthase activity (5-fluorouracil, FdUrd, and leucovorin) or thymidine kinase activity (5'-AdThd) (15–19). These agents were capable of enhancing the in vitro and in vivo incorporation of IdUrd and radiosensitization in various human tumor cell lines.

With the use of a similar rationale, a greater than additive effect on cytotoxicity has been shown by the combination of HU and ara-C, another pyrimidine nucleoside analogue (20–22). The sensitivity of human leukemia cells to ara-C is well correlated with ara-CTP levels and with incorporation into DNA (22, 23). Depletion of dCTP levels in cells by HU enhanced ara-C uptake and phosphorylation to ara-CTP. An increase in ara-C incorporation into DNA was also observed during coincubation with HU (23, 24).

Because of the possible inhibitory effects of HU on de novo dTMP synthesis (Fig. 1), HU may enhance the incorporation of IdUrd into DNA. We speculated that HU would result in an increased use of the salvage pathway through thymidine kinase. This hypothesis argues that HU could be an additional modulator for IdUrd incorporation and radiosensitization. In this study, we used a human bladder cancer cell line, 647V, to test the potential effects of HU on the modulation of IdUrd incorporation into DNA and IdUrd radiosensitization. Using concomitant exposures with noncytotoxic concentrations of HU, we report an increase in IdUTP pools and an increase in IdUrd-DNA incorporation and subsequent radiosensitization compared with those seen with IdUrd alone.

MATERIALS AND METHODS

Cell Culture

A human bladder cancer cell line, 647V, was used in this study as described previously (17). 647V cells were maintained in α-MEM ( Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 1 mM l-glutamine, 1 g/L nonessential amino acids, 1 g/L Na-pyruvate, and 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B.
and 1 mM nonessential amino acids (α-MEM+). Cultures were passed twice weekly and screened regularly for Mycoplasma contamination. 647V cells have a population doubling time of 24 h and a plating efficiency of 40–70% in our laboratory (17).

Chemicals and Drugs

Hydroxyurea, iododeoxyuridine, and other biochemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile (Burdick and Jackson, Muskegon, MI) was purchased from Baxter Scientific (Waukegan, IL). [6-3H]IdUrd (18 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA).

Hydroxyurea Cytotoxicity

Exponentially growing 647V cells were trypsinized, resuspended in cold α-MEM+, and counted. The cell suspension was serially diluted, and an appropriate number of cells were plated on 60-mm culture dishes (Corning, Inc., Corning, NY) containing 3 ml of α-MEM+. The dishes were then incubated for 4 h at 37°C to allow the cells to attach to the plate. The medium was then replaced with fresh α-MEM+ containing 0–1 mM HU. The dishes were incubated for 24 h at 37°C in the presence of the drug. After a 24-h incubation, the medium was then replaced with fresh drug-free α-MEM+. The dishes were then incubated for an additional 8–9 days at 37°C. Cells were then fixed and stained with 0.5% crystal violet in methanol:acetic acid (3:1, v/v). Colonies containing more than 50 cells were scored.

Flow Cytometry

Preparation of Cells for Analysis by Flow Cytometry. The nuclei of 647V cells were prepared by a modification of the technique described by Schutte et al. (25). Cells were pulsed with 20 μM BrdUrd for 15 min before each time point after 0 or 100 μM hydroxyurea exposure. The medium containing BrdUrd was then aspirated, and cells were washed with PBS-EDTA, trypsinized, and pelleted (300 × g, 5 min, 4°C). Cold PBS (0.7 ml) was added into each sample with gentle mixing. Then, 1.3 ml of cold 95% ethanol containing 0.5% Tween 20 was added dropwise into the cell suspension with gentle mixing. Cells were fixed at 4°C in the dark until the staining procedure was performed (within 7 days). PBS (5 ml) was then added to cells, and the cell suspension was pelleted (300 × g, 5 min, room temperature) to remove ethanol. The pellet was suspended in 0.5 ml of 0.4% pepsin in 0.1 N HCl and incubated for 30 min at room temperature in the dark. The nuclei were pelleted (600 × g, 5 min), resuspended in 1.5 ml of 2 N HCl, incubated for 30 min at 37°C, and shaken twice during incubation. HCl was removed by centrifugation (600 × g, 10 min). The pellet was washed twice with 5 ml of PBS-TB to neutralize the remaining HCl. The nuclei were then resuspended in 1 ml of RNase A solution (10 μg/ml in PBS-TB; Sigma) and incubated for 20 min in the dark at 37°C. RNase A solution was removed by centrifugation (600 × g, 5 min) and washed with 3 ml of PBS-TB. The nuclei were resuspended in 50 μl of PBS-TB, and 20 μl of anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) was added to the suspension. The nuclei were incubated overnight at 4°C in the dark. Three ml of PBS-TB were added to wash the pellet and then centrifuged (600 × g, 10 min). The pellet was resuspended in 0.2 ml of goat anti-mouse immunoglobulin-FITC conjugate (0.02 mg/ml; Sigma) and incubated for 20 min at room temperature in the dark. The nuclei were washed twice in 3 ml of PBS-TB, and the final pellet was resuspended in 0.5 ml of PBS-TB containing 50 μg/ml propidium iodide (Sigma). This suspension was stored at 4°C until analyzed by flow cytometry (within 48 h).

Flow Cytometric Analysis. The BrdUrd/DNA signal was analyzed with the use of a Becton Dickinson FACScan flow cytometer with 15 mW excitation at 488 nm. The FITC signal was collected through a 530/30 band pass filter on FL1, and the PI signal was assessed through a 650 long pass filter on FL3. The software used for acquisition and analysis was Lysis II (Ver. 1.1; Becton Dickinson). Doublets were excluded by analysis of two-dimensional plots of fluorescence pulse width versus area of the PI signal, and singlets only were counted for cell cycle distribution statistics. Windows were drawn to define cell populations in each cell cycle phase in contour plot or dot plot of FL1 versus FL3. The population with the BrdUrd-negative signal and 2N DNA content detected by PI is in G1. The population with the BrdUrd-negative signal and 4N DNA content is in G2-M. Those cells with BrdUrd-positive signal are in S phase. To determine the cells in early or late S phase, the region containing BrdUrd-positive events was divided in half. The half with less PI signal was defined as the early S-phase population, and the half with higher PI signal was defined as the late S-phase population. The percentage of the events in each window was calculated by the Lysis II program.

Thymidine Replacement

Exponentially growing 647V cells were incubated with 0–300 μM HU for 2 h. Then, 2 μM IdUrd was added, and the cultures were incubated for an additional 24 h (1 cell cycle) at 37°C. Cells were then washed with ice-cold PBS. The percentage of dThd replacement was determined with the use of a modificiation of the method of Belanger et al. (26). Cells resuspended in α-MEM+ were harvested by centrifugation at 650 × g for 10 min. The medium was removed, and the cell pellet was washed once with 5 ml of ice-cold PBS. The cells were recovered by centrifugation, then resuspended in 1 ml of water. One ml of 10% TCA was added to the cell suspension with vigorous mixing. The resultant precipitate was recovered by centrifugation for 10 min at 650 × g. The pellet was washed once in 1 ml of 10% TCA. The precipitate was then incubated for 90 min at 37°C in 1 ml of 0.25 N NaOH to hydrolyze the RNA. The DNA was precipitated by the addition of 5 ml of 10%...
TCA. Next, the DNA was suspended in 400 μl of a mixture containing 25 mm potassium phosphate buffer (pH 7.45), 2 mm magnesium chloride, 0.05 mg/ml DNase I, 0.3 unit/ml alkaline phosphatase, and 1.4 mg/ml phosphodiesterase. The mixture was incubated for 2 h at 37°C. Debris was removed by centrifugation for 10 min at 1000 × g. The supernatant was ultrafiltered through a Millipore Ultrafree-MC filtration unit (Millipore Corp., Bedford, MA). The ultrafiltrate was stored at −20°C for HPLC analysis.

**HPLC Analysis**

HPLC analysis of nucleosides was performed with the use of a Waters 600E solvent delivery system on a 3.9 x 300 mm μBondapak C18 reversed-phase column (Waters Associates, Milford, MA). Peak elution was monitored by UV absorbance with the use of a Waters Model 490E UV detector. Data analysis was performed with the use of a Waters Model 745 data module against authentic nucleoside standards.

IdUrd-DNA incorporation was determined with the use of a mobile phase that consisted of 100 mm sodium acetate (pH 5.45) containing 7% (v/v) acetonitrile at a flow rate of 2 ml/min. Peaks were detected at 290 nm. The percentage of dThd replacement was calculated as nmol IdUrd/(nmol dThd).

**Measurement of IdUTP Pools**

Cells were preincubated with 100 μM HU for 2 h. Twenty μM [6-3H]IdUrd (250 dpm/μmol) were added to a final concentration of 2 μM, and the dishes were incubated at 37°C for an additional 2–6 h. At 2-h intervals, the medium was aspirated, and the dishes were washed twice with ice-cold PBS. Then, 7 ml of ice-cold PBS was added to each dish, and the cells were detached from the dishes by scraping. The cells were transferred to a 15-ml conical centrifuge tube and placed on ice. Each dish was rinsed with 7 ml of ice-cold PBS and combined with the sample. Cells were then pelleted at 4°C by centrifugation for 10 min at 850 × g. The supernatant was removed by aspiration. Cells were mixed with 300 μl of ice-cold 0.5 n HClO4. The mixture was incubated on ice for 30 min. The cell debris was removed at 4°C by centrifugation for 10 min at 850 × g. The supernatant was then transferred to a 1.5-ml microcentrifuge tube. The pH of the supernatant was neutralized by vigorous mixing for 30 s with 2 volumes of trifluorochloroethanes-α-octylamine (2.5:1, v/v). The aqueous phase was recovered and transferred to a microcentrifuge tube. Ribonucleosides were degraded by periodate-methylamine treatment (27). The samples were lyophilized and stored at −80°C. The samples were redissolved in distilled H2O, and insoluble material was removed by centrifugation. Samples were injected onto a 4.7 x 235 mm Whatman VPS Partisil SAX as ion exchange column fitted with a Whatman guard cartridge at 45°C. Peaks were eluted by a linear 200–700 mM ammonium phosphate buffer gradient (pH 2.7). Fractions (0.3 ml) were collected, mixed with 5 ml of Poly-Fluo scintillation mixture (Packard Instrument Co., Meriden, CT), and counted in a Beckman LS-6000 scintillation spectrophotometer (Beckman Instruments, Fullerton, CA).

**Radiation Survival**

Cells were incubated in 0, 10, or 100 μM HU for 2 h at 37°C. Then, 2 μM IdUrd was added to the culture for an additional 24 h. This concentration of IdUrd results in minimal cytotoxicity in 647V cells (17) and is a clinically achievable plasma concentration with the use of a continuous intravenous infusion (13, 28, 29). Controls were cells treated with radiation alone or with a 24-h exposure to HU alone (0, 10, or 100 μM) or 2 μM IdUrd alone before irradiation. Cells were then trypsinized and resuspended in cold α-MEM+ alone. The cell suspension was then counted and serially diluted. Cells were irradiated with the use of a 137Cs irradiator at a dose rate of 7.01 Gy/min. After irradiation, cells were plated, incubated for 8–9 days, and scored as described above.

**Radiation-Drug Survival Analysis**

Two-stage Method of Cell Survival Analysis. We have reported a two-stage method for the analysis of cell survival sequences (30). Each dose-response sequence was first fitted to a modified form of the linear-quadratic model (30) by the equation: \( \log (\text{raw cell survival}) = \gamma - \alpha(D) - \beta(D)^2 \), where \( D \) = radiation dose (Gy). This parametric model is sufficiently flexible to fit the data well.

In the second stage of the two-stage method, the estimated parameters \( \alpha \) and \( \beta \) were treated as the data. The estimated intercept \( \gamma \) was not included in the analysis, because only shapes of the curves and not their intercepts are being compared.

The \( \alpha \) and \( \beta \) values were then analyzed with the use of a MANOVA to investigate the difference between the treatments. MANOVA is a statistical analysis that uses a generalized univariate ANOVA to handle multivariate responses; this method provides a unified alternative to testing differences among survival curves one parameter at a time. Therefore, the mean parameter vectors (\( \alpha \) and \( \beta \)) from pairs of whole-sequence treatments were tested by MANOVA to determine whether the mean parameter vector of one whole sequence is significantly different from another (30).

Testing for Interaction. Two agents are said to interact (after normalization to achieve equal intercepts) when the ratio of the survival curve for the combined agents and the survival curve for the controls is not equal to the product of the ratios of the survival curves for the single agents and the controls. The definition of interaction is a multivariate extension of the usual univariate test for additivity in the log scale. The presence of an interaction can be tested with the use of MANOVA (29).

**Calculation of SER.** Various definitions of radiosensitization have been used in the literature. In a previous in vitro study, we quantified radiosensitization by IdUrd by comparing the entire survival curves using the values of \( \alpha + \beta \) from the modified linear-quadratic fit for radiation survival as described above (30, 31). The simplest definition of SER is the ratio of \( \alpha + \beta \) with pretreatment by drug (HU, IdUrd, and HU + IdUrd) as the numerator and \( \alpha + \beta \) with radiation alone as the denominator.

**RESULTS**

Cytotoxicity of HU on 647V Cells. 647V cells were exposed to concentrations from 0 to 1 mM HU for one cell doubling time (24 h) and assayed by in vitro clonogenic survival (Fig. 2). HU exposure up to 100 μM produced no cytotoxicity (SF = 0.995 ± 0.029), whereas 300 μM HU was modestly cytotoxic in 647V cells (SF = 6.47 ± 0.186). Survival of 647V cells was significantly decreased after exposure to 1 mM HU. Less than 10% of cells survived after 24 h of exposure (SF = 0.070 ± 0.032).

**Effect of HU on Cell Cycle Distribution of 647V Cells.** Because it is well recognized that HU can synchronize cells in early S phase, in which cells may show enhanced radiosensitivity compared with
Sensitivity of 647V Cells. Cells were incubated with 0, 10, or 100 pM HU for 2 h, followed by 0 or 2 pM IdUrd for an additional 24 h. The 2-fold increase in IdUTP pools.

Combined treatment of HU and IdUrd resulted in an approximately 2-fold when 100 pM HU (14.6 ±1.2%) was used. A higher concentration of HU (300 ± M) did not further enhance IdUrd-DNA incorporation by this higher concentration of HU.

Effect of HU on IdUrd Incorporation into DNA of 647V Cells. The cells were initially exposed to various concentrations of HU for 2 h, and then 2 μM IdUrd was added to the culture for an additional 24 h. The incorporation of IdUrd into the DNA was determined and represented by the percentage of thymidine replacement. The level of thymidine replacement after a 24-h exposure to 2 μM IdUrd alone was 8.57 ± 0.40%. Thymidine replacement increased when the cells were pretreated with HU concentrations of 10, 30, 100, and 300 μM for 2 h and then coincubated with 2 μM IdUrd and HU for an additional 24 h (Fig. 4A). The maximum increase in thymidine replacement was approximately 2-fold when 100 μM HU (14.6 ± 1.2%) was used. A higher concentration of HU (300 μM) did not further enhance IdUrd-DNA incorporation. This effect may be caused by the inhibition of DNA replication by this higher concentration of HU.

Effect of HU on IdUTP Pool Size in 647V Cells. Our hypothesis is that the enhancement of IdUrd incorporation by HU results from inhibiting the de novo deoxyribonucleotide synthesis and stimulating the salvage pathway synthesis (Fig. 1). As a result, concomitant exposure to HU and IdUrd should increase IdUMP conversion and IdUTP pools (Fig. 1). To test the hypothesis, IdUTP pools were compared between cells that were exposed to 2 μM IdUrd alone and cells that were pretreated with 100 μM HU for 2 h and then coincubated with 2 μM IdUrd and HU for additional 2–6 h. The IdUTP pools in cells without HU exposure increased from 3.03 ± 0.31 pmol/10^6 cells at 2 h to 4.79 ± 0.79 pmol and 4.20 ± 0.26 pmol/10^6 cells after 4- and 6-h exposure, respectively (Fig. 4B). IdUTP pools in cells coincubated with HU and IdUrd were 6.66 ± 0.54, 8.19 ± 0.77, and 7.90 ± 0.39 pmol/10^6 cells for 2, 4, and 6 h, respectively. Thus, the combined treatment of HU and IdUrd resulted in an approximately 2-fold increase in IdUTP pools.

Effect of Preirradiation Exposure to HU and IdUrd on Radiosensitivity of 647V Cells. Cells were incubated with 0, 10, or 100 μM HU for 2 h, followed by 0 or 2 μM IdUrd for an additional 24 h. The radiation survival was then determined by clonogenic survival (Fig. 5). Although 10 μM HU alone did not radiosensitize the 647V cells (P = 0.884), 100 μM HU significantly enhanced radiosensitivity (P = 0.014; Fig. 5A). The SER comparing α + β values for pretreatment with 100 μM HU compared with radiation alone is 1.15 ± 0.06.

The effects of HU on IdUrd radiosensitization are presented in Fig. 5B. Two μM IdUrd was capable of radiosensitizing 647V cells when compared with control (P = 0.0002) with a SER of 1.27 ± 0.07. Although concomitant incubation with 10 μM HU and 2 μM IdUrd did not enhance radiosensitivity in 647V cells versus 2 μM IdUrd alone (P = 0.390), 100 μM HU and 2 μM IdUrd enhanced the radiosensitivity of 647V cells when compared with IdUrd alone (P = 0.0001) or 100 μM HU alone (P = 0.0001). Analysis of the survival curves with
the use of the method of Lindstrom et al. (30) demonstrated a greater than additive response to radiation for the combination of 100 μM HU and 2 μM IdUrd (P = 0.01). The observed SER was 1.76 ± 0.09 compared with the expected SER of 1.36.

**DISCUSSION**

The rationale for using a S-phase sensitizer such as IdUrd is to specifically radiosensitize highly dividing and poorly radiosensitive tumor cells in a way that distinguishes them from the surrounding slowly proliferative or nonproliferative normal tissues (33). The radiosensitizing effect of IdUrd is directly associated with the percentage of thymidine replacement (2, 3, 8–12). Therefore, modulation of IdUrd incorporation into cells by another chemotherapeutic agent during radiotherapy is a potentially useful approach to enhance the radiosensitivity of tumor cells. Several strategies have been pursued to modulate the incorporation of IdUrd into DNA in human tumor cells. In our laboratory, we have evaluated the modulation of IdUrd incorporation into DNA by 5'-AdThd, a noncytotoxic modulator of thymidine kinase activity, in 647V cells in vitro (17). Studies on the effect of 5'-AdThd on the modulation of IdUrd incorporation in vivo are ongoing. FdUrd and leucovorin have also been shown to enhance IdUrd-DNA incorporation and subsequent radiosensitization of colon cancer cell lines, presumably through inhibition of endogenous thymidine synthesis (16, 19). However, because of the greater systemic toxicity of the combination, no therapeutic gain was observed when IdUrd and FdUrd were used clinically in a Phase I study (29). At present, we are evaluating the combination of leucovorin and iododeoxyuridine in a Phase I trial (34).

In this study, we were able to demonstrate a direct correlation of the increases of IdUrd incorporation, IdUTP pools, and radiosensitization to incubation with a noncytotoxic dose of HU (100 μM) for one cell population doubling time (24 h) combined with a clinically relevant concentration of IdUrd (2 μM) (13, 27, 28). Our rationale involves using noncytotoxic doses of HU to modulate IdUrd incorporation through the inhibition of de novo dTMP synthesis, resulting in enhanced use of thymidine kinase to synthesize dTMP (Fig. 1). Because IdUrd competes with thymidine for conversion to the monophosphate, greater use of thymidine kinase facilitates the conversion of IdUrd to IdUMP and, after further phosphorylation, to IdUTP. In 647V cells, exposure to 100 μM HU resulted in a 2-fold increase in IdUTP pools within 2 h (Fig. 4B). The increase in IdUTP pools in HU-treated cells measured over the initial 6 h correlated with a similar increase in dTTP replacement by IdUrd into DNA (Fig. 4A) and with enhanced IdUrd radiosensitization with a 24-h exposure (Fig. 5B).

This hypothesis about HU-IdUrd interaction is supported by studies investigating the effect of HU on the metabolism of deoxyribonucleotide triphosphates. Modulation of dNTP pools by HU has been evaluated in several cell lines in vitro (35–41). Unexpectedly, only purine deoxyribonucleotide triphosphate pools were decreased in the presence of HU. dTTP pools were increased by HU in the most of the cell lines studied. Bianchi et al. (5) reported the modulation of dNTP pools by HU in exponentially growing 3T6 cells (5). In 3T6 cells, ribonucleotide reductase activity was completely blocked by 3 mM HU, and an increase of dTTP pools was observed within 5 min after addition of HU. An increase in the uptake and phosphorylation of deoxyuridine or thymidine and deamination of dCTP accounted for the increase of dTTP pools. Because DNA synthesis was inhibited within 5 min after administration of HU, decreased incorporation of dTTP into DNA also contributed to the accumulation of dTTP pools. This also implied that feedback inhibition of thymidine kinase did not recover fast enough to maintain the constant dTTP pools in the presence of HU.

It has been shown previously that a high concentration of HU (2 mM) enhanced the incorporation of [3H] thymidine into the acid-soluble fraction in the cells, suggesting that HU can modulate the salvage pathway metabolism of thymidine (42). However, 2 mM HU reduced the incorporation of [3H] thymidine into genomic DNA, suggesting that high HU concentrations can inhibit DNA synthesis/repair. Low-dose HU (100 μM) in combination with clinically relevant concentrations of IdUrd (2 μM) may, therefore, radiosensitize cells by increasing IdUTP for DNA incorporation and decreasing repair of the IdUrd-enhanced radiation-induced DNA damage (33).

It has been suggested that HU alone is a potential radiosensitizer (20, 21, 32). It is known that HU inhibits ribonucleotide reductase activity and synchronizes cells in early S phase. Because of the greater sensitivity to radiation in early S phase in Chinese hamster cells, HU may enhance radiosensitivity by cell cycle synchronization alone (41). We found that the radiosensitivity of 647V cells was significantly enhanced by previous incubation with low-dose HU (100 μM) for one cell cycle time (24 h) (Fig. 5A). Using bromodeoxyuridine and PI labeling, we analyzed the cell cycle distribution during a 24-h exposure to 100 μM HU. The early S-phase population increased and reached a maximum (43.1% early S phase) after 12-h exposure to 100 μM HU but returned to normal (29.9%) after 24 h of exposure (Fig. 3). It is interesting that 647V cells irradiated after a 12-h exposure to 100 μM HU showed no change in radiosensitivity (P = 0.440), whereas a longer (24-h) exposure to 100 μM HU did slightly radiosensitize 647V cells (Fig. 5A). These data indicate that HU may enhance the radiosensitivity of 647V cells by a mechanism other than cell cycle synchronization in early S phase.

All of the significant effects on IdUrd metabolism and radiosensitization observed in these studies were found with the use of noncytotoxic concentrations of HU. Because the ultimate goal of the study is to provide a potential alternative strategy in radiotherapy, the differential radiosensitivity between tumor cells and surrounding normal tissue is of great concern. By using noncytotoxic concentrations of HU, we may further differentiate the incorporation of IdUrd in tumor cells from surrounding normal tissue without increasing the toxicity to normal tissues. This implies a potential translational application in the clinic, although it will require additional study in vivo to demonstrate its feasibility.

The modulation of IdUrd incorporation by high-dose HU in bone marrow and tumor cells has been evaluated in a recent Phase I study (43). This study tested the concept of "reverse-role chemotherapy" proposed by Bagshawe (44), in which HU is used to temporarily suppress DNA synthesis in dose-limiting normal tissues such as bone marrow. Subsequently, a cytotoxic nucleotide could be administered and preferentially taken up by tumor cells in which DNA synthesis had not been inhibited as a result of de novo or acquired drug resistance. However, in this Phase I study, IdUrd incorporation was equally reduced in bone marrow and tumor cells. Because the mean plasma concentration of HU at the time of IdUrd administration was 1.7 nM, DNA synthesis in bone marrow and tumor cells may have been severely inhibited. Although HU enhanced the accumulation of IdUTP in the normal and tumor cells, cessation of DNA synthesis could lead to inhibition of IdUrd incorporation into DNA. In our in vitro study, lower (>1 log) concentrations of HU were able to enhance the incorporation of IdUrd into DNA. A lower dose of HU may be useful to differentiate the incorporation of IdUrd into bone marrow and tumor cells in vivo.

**REFERENCES**


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