Effects of 5'-Aminothymidine and Leucovorin on Radiosensitization by Iododeoxyuridine in Human Colon Cancer Cells

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ABSTRACT
We examined the effects of two biochemical modulators, 5'-aminothymidine (5'-AdThd) and leucovorin (LV), on the in vitro incorporation of iododeoxyuridine (IdUrd) into DNA and its subsequent radiosensitization in two human colon cancer cell lines, HT 29 and HCT 116. 5'-AdThd is a modulator of thymidine kinase activity while LV is an essential cofactor for thymidylate synthase activity. In HT 29 cells, the combination of 5'-AdThd (10 μM) and IdUrd (1–10 μM) resulted in a significant increase in IdUrd triphosphate pools and in IdUrd-DNA incorporation. Coadministration of LV (10 μM) with IdUrd (1–10 μM) resulted in a significant decrease in thymidine triphosphate pools and a comparable increase in IdUrd-DNA incorporation as the combination of 5'-AdThd + IdUrd. The increase in radiosensitization by clonogenic survival with either combination was a direct linear function with the percentage of IdUrd-DNA incorporation. For HCT 116 cells, however, the results were different. While 5'-AdThd + IdUrd resulted in an increase in IdUrd triphosphate and percentage of IdUrd-DNA incorporation, significant cytotoxicity was noted. The radiosensitivities of HCT 116 cells treated with 5'-AdThd + IdUrd was not a linear function above 25% IdUrd-DNA incorporation. Also, no increase in IdUrd-DNA incorporation or radiosensitization was observed with LV + IdUrd although LV enhanced the decrease in thymidine triphosphate pools by IdUrd treatment. These results indicate heterogeneity in the response of different colon cancer cells to these modulators which may be related to the regulation of deoxynucleotide metabolic enzymes.

INTRODUCTION
The halogenated pyrimidines, IdUrd and BrdUrd, have been used in a variety of recent Phase I and II clinical trials to test the efficacy of their sensitizing properties for ionizing radiation (reviewed in Ref. 1) and for radiomimetic agents such as bleomycin (2, 3). Clinical radiosensitization has been suggested in several trials in patients with anaplastic astrocytomas, high-grade unresectable sarcomas, and some locally advanced head and neck cancers (4–8). However, only limited success has been observed in patients with glioblastoma multiforme (7, 9, 10). A Phase III trial is ongoing by the Northern California Oncology Group and the Radiation Therapy Oncology Group in patients with anaplastic astrocytoma to confirm the role of these halogenated pyrimidine analogues as radiosensitizers compared to irradiation alone (6).

In experimental systems, a positive correlation exists between the amount of DNA incorporation for these compounds and the extent of radiosensitization observed (1). This correlation is also evident in the clinic, because the efficacy of these compounds as radiosensitizers is related to both the labeling index (number of cells that have taken up the sensitizer) and the percentage of DNA incorporation in individual tumor cells (1, 4). Indeed, the limited efficacy of IdUrd and BrdUrd in sensitization of clinically poorly radioresponsive tumor types such as glioblastoma multiforme may be best correlated with the limited incorporation of the analogues into tumor cells (4). Recent clinical trials attempting to increase the DNA incorporation in these tumor types have included alterations in the route of administration (intraarterial versus i.v.) or combined administration of the halogenated pyrimidines with cytotoxic antimetabolites that modulate pyrimidine nucleoside pathways (1). Neither approach has resulted in clinically significant progress to date, as the former approach involves a higher risk of catheter-related injury to normal vascular and the latter involves increases in systemic normal tissue toxicities that limit the dose rate and total dose of the sensitizer (1, 11–14).

In this study, we examined, on a biochemical level, the effect of modulating the activities of two pathways important in the incorporation of IdUrd into DNA using coadministration of IdUrd with two potentially less toxic modulators, 5'-AdThd and LV. Human colon cancer cells are the experimental system chosen for this study because of our continuing laboratory and clinical interest in the development of radiosensitizing treatment regimens for advanced gastrointestinal cancers (14–16). Understanding more about the processing of IdUrd in these cells and finding ways to improve the efficiency of IdUrd incorporation into tumor cell DNA may help to improve treatment strategies for halogenated pyrimidines as radiosensitizers in the clinic. Indeed, based on these in vitro data, we are conducting a Phase I study of the coadministration of IdUrd and LV as a radiosensitizing regimen in this patient population (16). Preliminary results suggest that LV can enhance IdUrd-DNA incorporation as demonstrated in patients' peripheral granulocytes.

The uptake and incorporation of IdUrd into cellular DNA are dependent on the nucleoside salvage pathway of a cell. Once internalized, IdUrd is phosphorylated to its monophosphate form (IdUMP) by TK, as detailed in Fig. 1. The addition of two
more phosphate groups occurs rapidly thereafter, and IdUTP is then used by DNA polymerase in DNA replication (17). There are two possible rate-limiting steps in this process. The first occurs at TK, which is feedback inhibited by IdUTP and by its naturally occurring inhibitor dTFP (17). The second occurs at DNA polymerase, at which point IdUTP is assumed to be in direct competition with dTTP for incorporation into DNA. dTTP is synthesized by two routes. The first is exogenous dThd which is processed through TK in the nucleoside salvage pathway, as outlined for IdUrd in Fig. 1. The second is de novo production of dTTP through TS, which converts dUMP to dTMP. dTTP production through the TK pathway is not likely to be a major source of competing dThd, as extracellular levels of dThd generally do not exceed 0.2 μM in humans (18). In addition, the presence of much higher plasma levels of IdUrd, as would occur during a continuous iv. infusion (1–5 μM), would effectively compete with exogenous dThd for the active site of TK (1). However, a second source of dTTP, through the TS pathway, can remain operative under these conditions and is likely the major source of dTTP that would compete with IdUTP for incorporation into DNA.

Two possible strategies emerge to increase the relative amount of IdUTP produced in a cell: (a) increase the activation of IdUrd through TK or (b) decrease the formation of dTTP through TS. To increase the activation of IdUrd to its phosphorylated forms, TK activity must be increased, since it is the rate-limiting step of this nucleoside salvage pathway. Simply increasing IdUrd levels to saturate enzyme activity may not result in more incorporation, since TK is strongly feedback inhibited by the triphosphate end product, IdUTP (19); i.e., high levels of IdUrd will result in high levels of IdUTP that will, in turn, reduce TK activity by binding at an allosteric regulatory site. A better strategy is to reduce the feedback inhibition of TK by the triphosphate end product. This has been accomplished in human tumor cells with 5'-AdThd, a thymidine analogue that competes with dTTP (or IdUTP) at an allosteric site of regulation on TK. Previous studies have shown that 5'-AdThd increases the uptake and phosphorylation of dThd and its analogues (including IdUrd and FdUrd) in various cell types (20–22). In our laboratory, this increased uptake has been associated with increased incorporation of IdUrd into the DNA of human bladder cancer cells (23).

Decreasing TS activity in order to increase IdUrd incorporation into DNA has already been attempted in the laboratory and clinic. In vitro studies showed that cotreatment with TS inhibitors such as FdUrd or 5-fluorouracil increased IdUrd DNA incorporation, resulting in enhanced radiosensitization (24–26). Unfortunately, this strategy essentially failed in recent clinical trials due to the increased systemic toxicity of the combinations (1, 12, 13). However, a related in vitro study demonstrated that the monophosphate form of IdUrd itself is a weak inhibitor of TS. This inhibition is enhanced by the addition of LV, a precursor of methylene tetrahydrofolate, which is the cofactor required by TS for the transfer of a methyl group to dUMP to form dTMP (Fig. 1; Ref. 27). The halogenated pyrimidines are false substrates for TS; i.e., these compounds can bind to TS and form the same enzyme-substrate-cofactor complex as the natural substrate. However, the transfer of a methyl group to the substrate cannot be accomplished due to the presence of the halogen. Because equilibrium favors the enzyme-substrate-cofactor complex, its dissociation is slow and TS remains bound to the monophosphate and essentially inhibited during this association.
By expanding the folate cofactor pool, LV may thus slow the
dissociation of the complex by a simple mass-action effect (28).
TS may also work in the reverse direction without folate to
dehalogenate IdUMP. Coadministration of LV to increase folate
pools may keep TS bound in an inactive complex for a longer
period of time, thereby slowing the catabolism (by dehalogenation)
of IdUMP (29).

As single agents, both 5'-AdThd and LV are relatively
nontoxic (27, 28, 30), making these compounds potentially good
candidates for modulators in in vivo systems and in the clinic.
Thus, we examined the effects of 5'-AdThd or LV on the
intracellular production of deoxyribonucleotide triphosphates
and how these effects might result in enhanced IdUrd-DNA
incorporation and radiosensitization in the human colon cancer
cell lines HT 29 and HCT 116.

MATERIALS AND METHODS

Materials. IdUrd was obtained from Calbiochem (La
Jolla, CA). [6-3H]IdUrd (18 Ci/mmol) was obtained from
Moravek Biochemicals (Brea, CA). 5'-AdThd, LV, and all other
materials were obtained from Sigma Chemical Company (St.
Louis, MO) unless indicated otherwise.

Cell Culture. HT 29 (31) and HCT 116 (32) human
colon cancer cells were obtained from American Type Culture
Collection. The cells were maintained in α-MEM (GIBCO,
Grand Island, NY) supplemented with 10% v/v FBS (GIBCO),
25 mM HEPES buffer, and 5 μg/ml gentamicin (GIBCO). HT 29
cells have a doubling time of 24 h and were passed once a week
at low density (4 × 10⁴ cells/100-mm dish) for optimal growth.
HCT 116 cells have a shorter doubling time of approximately
18 h under these conditions and were passed biweekly at moder-
ate density (1 × 10⁵ cells/100-mm dish) for optimal growth.
When used for an experiment, the cells were plated into α-MEM
supplemented with 10% v/v dialyzed FBS, 0.2 μm dThd, 25 mM
HEPES buffer, and 5 μg/ml gentamicin. The cells were allowed
to attach overnight and drugs were administered the following
day. Growth in medium supplemented with dialyzed serum did
not differ significantly from that observed in medium supple-
mented with nondialyzed serum. dThd (0.2 μM) was added to
the medium containing dialyzed serum to mimic levels of dThd
observed in human plasma (18). When exposure to drug(s) was
extended to two cell doubling times, medium and drug(s) were
replaced after one doubling. Procedures involving IdUrd treat-
ment were carried out under low-wattage yellow light to mini-
mize sensitization of treated cells to ambient fluorescent light
(33).

Extraction of Intracellular Deoxynucleotides. For
these experiments, HT 29 cells were plated into 150-mm dishes
containing 20 ml medium and HCT 116 cells were plated in
100-mm dishes with 10 ml medium. The assay began upon
addition of [6-3H]IdUrd (0–10 μM at a specific activity of 200
dpm/μmol) to the medium. The dishes were incubated at 37°C
for 1 h. Medium was aspirated and the dishes were washed twice
with ice-cold Dulbecco’s PBS (GIBCO). PBS (7 ml) was then
added and the cells were detached from the dish by scraping.
The cells were transferred to a 15-ml conical centrifuge tube
and placed on ice. Each dish was then rinsed with another 7 ml PBS
and combined with the appropriate sample. The cells were
pelleted by centrifugation at 4°C (200 × g). The PBS was
removed by aspiration and the cells mixed with 300 μl (HT 29)
or 200 μl (HCT 116) of ice-cold 0.5 M perchloric acid.
The mixture was incubated on ice for 30 min. Cellular debris was
removed by centrifugation (850 × g at 4°C) and the supernatant
was transferred to a 1.5-ml microcentrifuge tube. The pH of the
supernatant was neutralized by vigorous mixing (30 s) with
2 volumes of trifluorotrichloroethane:n-octylamine (2.5:1; Al-
drich Chemical Company, Milwaukee, WI). The upper (aque-
ous) layer was recovered and transferred to a clean 1.5-ml
microcentrifuge tube.

Ribonucleosides were then degraded in each sample by
periodate-methylamine treatment. Briefly, 20 μl 0.5 M sodium
periodate (Aldrich) was added to the cell extract followed by 25
μl 4 M methylamine-HCl, pH 7.5 (Aldrich). The samples were
mixed thoroughly and incubated at 37°C for 30 min. Ten μl of
1 M rhamnose were added (to destroy any remaining periodate)
and the samples were placed on ice. The samples were frozen in
an ethanol-dry ice bath and lyophilized to dryness. The samples
were redissolved in 210 μl dH₂O and analyzed by HPLC for
quantitation.

HPLC Determination of Intracellular Concentrations
of dTTP and IdUTP. Treated cell extracts were processed using
a Waters HPLC system (Waters, Bedford, MA). Nucleo-
"tides were separated on a Whatman VWS Partisil SAX ion
exchange column (4.7 × 235 mm) using a biphasic gradient
program beginning with a linear gradient from 100% buffer A
(200 mM ammonium phosphate, pH 2.7) to 100% buffer B (700
mM ammonium phosphate, pH 2.7) over 30 min followed by
isocratic flow of 100% buffer B for 10 min, which was then
followed by a linear gradient from 100% buffer B to 100%-
buffer A over 15 min (flow rate, 2 ml/min; column temperature,
45°C). dTTP was detected at 267 nm and IdUTP was detected
at 288 nm. Under these conditions, dTTP eluted at 23.0 min and
IdUTP eluted at 25.1 min. dTTP was quantified by peak heights
against a curve of authentic standards. IdUTP was quantified by
scintillation spectrophotometry of 0.3-ml fractions that were
mixed with 5 ml Poly-Fluor Scintillation Cocktail (Packard
Instrument Co., Meridian, CA) using a Beckman LS-6000 scin-
tillation counter.

Determination of Percentage of dThd Replacement
by IdUrd. Cells were plated and treated with the drug in 100-mm
dishes with a cell density of 0.5 to 1 × 10⁶ cells/dish. Percent-
age of dThd replacement by IdUrd was measured by a modifi-
ation of the technique of Belanger et al. (34) as described
previously (35).

Radiation Survival. Radiation survival after treatment
with a single drug (IdUrd, 5'-AdThd, or LV) or a combination
(IdUrd with 5'-AdThd or LV) was determined as described
previously (35). Briefly, cells were plated and treated with
IdUrd (1–10 μM), 5'-AdThd (10 μM), LV (10 μM), and varying
concentrations of IdUrd (1–10 μM) ± 5'-AdThd or LV (10 μM)
for 1 or 2 cell doubling times in 160-cm² flasks. After drug
exposure, the cells were trypsinized, pelleted, and then resus-
pended in medium (supplemented with nondialyzed serum) at a
density of 3–6 × 10⁵ cells/ml. The cell suspension was distributed
in 4-ml aliquots among seven 25-cm² flasks which were
then sealed, put on ice, and kept in the dark. One flask remained
unirradiated and served as a control to determine plating effi-

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Table 1  Effect of pretreatment with 10 μM of 5'-AdThd or LV for one or two cell doubling times on the plating efficiencies and radiosensitivity (as measured by α) of HT29 and HCT 116 cells

<table>
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<tr>
<th></th>
<th>HT29</th>
<th></th>
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<th>HCT116</th>
<th></th>
<th></th>
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<tr>
<td>Cell doubling</td>
<td>Control</td>
<td>5'-AdThd</td>
<td>LV</td>
<td>Control</td>
<td>5'-AdThd</td>
<td>LV</td>
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<tr>
<td>PE</td>
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<td>0.393 ± 0.035</td>
<td>0.489 ± 0.004</td>
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<td></td>
<td>2</td>
<td>0.442 ± 0.032</td>
<td>0.402 ± 0.035</td>
<td>0.408 ± 0.027</td>
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<tr>
<td>α</td>
<td>1</td>
<td>0.075 ± 0.019</td>
<td>0.092 ± 0.035</td>
<td>0.083 ± 0.006</td>
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<tr>
<td></td>
<td>2</td>
<td>0.079 ± 0.033</td>
<td>0.092 ± 0.035</td>
<td>0.083 ± 0.006</td>
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<td></td>
</tr>
<tr>
<td>PE</td>
<td>1</td>
<td>0.705 ± 0.069</td>
<td>0.635 ± 0.056</td>
<td>0.728 ± 0.132</td>
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<td></td>
<td>2</td>
<td>0.598 ± 0.071</td>
<td>0.587 ± 0.071</td>
<td>0.607 ± 0.088</td>
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<tr>
<td>α</td>
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<td>0.473 ± 0.065</td>
<td>Not done</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.496 ± 0.057</td>
<td>0.496 ± 0.057</td>
<td>Not done</td>
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</tr>
</tbody>
</table>

* Data presented as mean ± 1, SE.
* PE, plating efficiency.

RESULTS

The results for nucleotide pools, DNA incorporation, and radiosensitization determinations were obtained using 10 μM 5'-AdThd or LV. These doses were not toxic when the modulator was administered as a single agent for one or two cell doubling times (in terms of plating efficiency, Table 1). Also, exposure to HT 29 and HCT 116 cells to 10 μM 5'-AdThd or LV alone for one or two cell doubling times did not alter radiation survival using the LQ values for α (Table 1). The effects of either 10 μM 5'-AdThd or LV and varying doses of IdUrd (0.5–10 μM) on the plating efficiency of HT 29 and HCT 116 cells for one or two cell doubling times are given in Tables 2 and 3. Measurements of dUTP and dTTP pool levels in HT 29 cells 1 h after addition of IdUrd ± 10 μM 5'-AdThd or LV to the culture are shown in Fig. 2. Data in Fig. 24 indicate that 5'-AdThd in combination with IdUrd (■) generally increased dUTP pools compared to IdUrd administered alone (●). A comparison of 5'-AdThd effect on dUTP levels following a 1-h coincubation with 0 or 5 μM IdUrd was significant (P < 0.007). However, 10 μM 5'-AdThd did not appear to affect dTTP pool levels, as shown in Fig. 2B (■; P = 0.5). Coadministration of 10 μM LV elicited different responses; in combination with IdUrd, LV did not increase dUTP pools (Fig. 2A, ▲), but did decrease dTTP pools to nearly undetectable levels (P < 0.005 with 0 versus 5 μM IdUrd; Fig. 2B, ▲).

The effect of coadministration of 10 μM 5'-AdThd on the incorporation of IdUrd into the DNA of HT 29 cells is shown for one and two cell cycles of exposure (Fig. 3). The curves differed statistically by MANOVA analysis for both exposure periods (P < 0.001); however, the shift induced by 5'-AdThd was not consistent over the entire range of doses at 24 h. This was evident in the pools data as well (Fig. 2). The largest enhancement of percentage of dTTP replacement occurred between 1 and 5 μM IdUrd in combination with 5'-AdThd, where IdUrd DNA incorporation reached an apparent maximum at 43%. In the other six flasks were irradiated on ice with increasing doses of 250-kV X-rays generated by a Philips TR 250 orthovoltage X-ray machine at a dose rate of 1.6 Gy/min. Flasks remained on ice until cells were plated at various densities in 60-mm dishes containing 5 ml medium. Three dishes were plated for each dilution density. The dishes were incubated for 10 days (for the HCT 116 cells) or 14 days (for the HT 29 cells), then the colonies were stained with crystal violet and counted. A colony was defined as containing at least 50 cells. Surviving fractions were calculated and linear-quadratic fits were accomplished using the FIT program (version 2.0) for analysis of cellular survival data as described (35).

Statistical Analyses. Differences between the treatments were investigated using MANOVA as implemented in the SAS GLM procedure as described in detail (2). Experiment number was included in the MANOVA model to adjust for experiment-to-experiment variability. MANOVA is a generalization of ANOVA (and the t test) appropriate for multivariate responses. MANOVA provides a unified alternative to testing differences in 60-mm dishes containing 5 ml medium. Three dishes were plated for each dilution density. The dishes were incubated for 10 days (for the HCT 116 cells) or 14 days (for the HT 29 cells), then the colonies were stained with crystal violet and counted. A colony was defined as containing at least 50 cells. Surviving fractions were calculated and linear-quadratic fits were accomplished using the FIT program (version 2.0) for analysis of cellular survival data as described (35).

The LQ model used and interpreted in this study is based in part on a theory of dual radiation action (36), which asserts that a yield of biologically relevant lesions is proportional to the square of the specific energy absorbed in a specific site. This model can describe the dose-effect relationships for a range of radiation qualities. In a recent in vitro study of the interaction of IdUrd and radiation on clonogenic survival in both HT 29 and HCT 116 cell lines, we reported a direct linear relationship of radiosensitivity (as measured by α) of HT29 and HCT 116 cells.
Fig. 4, the effect of 10 μM LV on incorporation of IdUrd into HT 29 DNA is shown for one and two cell cycles of exposure (Fig. 4). Enhancement by LV was statistically significant for both exposure periods (P < 0.001) over most of the dose range (1–10 μM) in a pattern similar to that for the reduction in dTTP pools in Fig. 2B. The maximum percentage of dThd replacement value was only slightly lower than that observed for 10 μM 5′-AdThd (40% as compared to 43%). Thus, both modulators are effective in increasing IdUrd DNA incorporation in HT 29 cells. The combination of 10 μM 5′-AdThd and 10 μM LV with 2 μM IdUrd was tested in the HT 29 cells. Measurements of the percentage of dThd replacement indicated that no additional IdUrd DNA incorporation was observed using the 5′-AT-LV combination (29.5 ± 1.1%) compared to that achieved with 10 μM 5′-AdThd alone (29.0 ± 1.1%). This combination was not tested in HCT 116 cells.

In the second human colon cancer cell line, HCT 116, the same pools and DNA incorporation measurements were made using the same dose range of IdUrd in combination with either 10 μM 5′-AdThd or 10 μM LV. Compared to the HT 29 cells, deoxyribonucleotide pools in HCT 116 cells are quite large, perhaps due to a different complement of enzymes involved in pyrimidine metabolic pathways in these cells. The deoxyribonucleotide pool measurements shown in Fig. 5A indicate that 10 μM 5′-AdThd effectively increased IdUTP concentrations in these cells over the majority of the dose range of IdUrd (■; P = 0.0001 with 0 versus 5 μM IdUrd). Ten μM LV also appeared to increase IdUTP levels at the lower doses (≤5 μM) of IdUrd (▲; P = 0.01 with 0 versus 5 μM IdUrd). In Fig. 5B, coadministration of 10 μM 5′-AdThd showed no consistent effect on dTTP concentrations (■), and 10 μM LV appeared to decrease dTTP levels only at low doses of IdUrd (≤2 μM; ▲).

As was the case for HT 29 cells, the IdUrd DNA incorporation data for HCT 116, shown in Fig. 6, reflects the pools data; 10 μM 5′-AdThd increased IdUrd DNA incorporation at both one and two cell cycles of exposure (Fig. 6, A and B, P = 0.0001 and 0.02, respectively). However, the toxicity of IdUrd, in terms of plating efficiency, was also significantly increased when 10 μM 5′-AdThd was coadministered (Table 3). In contrast, 10 μM LV showed no significant effect on IdUrd DNA incorporation for one or two cell cycles of coexposure, as shown in Fig. 7, A and B (P = 0.95 and 0.43, respectively), despite the apparent reduction in dTTP pools at low IdUrd concentrations (≤2 μM; Fig. 5B, ▲). The ineffectiveness of 10 μM LV as a modulator in HCT 116 cells may indicate that these cells have high intracellular levels of folates under the culture conditions used in these studies, thus TS inhibition may not have been increased by the addition of 10 μM LV. Therefore, IdUrd DNA incorporation levels would remain similar to those obtained with IdUrd alone.

### Table 2
Plating efficiencies (±SE) for HT 29 cells after continuous exposure for one (24-h) or two (48-h) cell doubling times to IdUrd alone or in combination with 10 μM 5′-AdThd or 10 μM LV

<table>
<thead>
<tr>
<th>[IdUrd] (μM)</th>
<th>IdUrd alone</th>
<th>IdUrd + 10 μM 5′-AdThd</th>
<th>IdUrd + 10 μM LV</th>
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<tr>
<td>24-h continuous exposure</td>
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<tr>
<td>0.5</td>
<td>0.464 ± 0.049</td>
<td>0.439 ± 0.069</td>
<td>0.368 ± 0.080</td>
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<tr>
<td>1</td>
<td>0.539 ± 0.051</td>
<td>0.343 ± 0.062</td>
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<td>2</td>
<td>0.381 ± 0.020</td>
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<td>5</td>
<td>0.492 ± 0.053</td>
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<td>10</td>
<td>0.382 ± 0.029</td>
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<tr>
<td>48-h continuous exposure</td>
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<tr>
<td>0.5</td>
<td>0.390 ± 0.070</td>
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<tr>
<td>5</td>
<td>0.235 ± 0.034</td>
<td>0.043 ± 0.001</td>
<td>0.095 ± 0.013</td>
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<tr>
<td>10</td>
<td>0.169 ± 0.017</td>
<td>0.034 ± 0.003</td>
<td>0.065 ± 0.007</td>
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*Control plating efficiency averaged 0.453 ± 0.030.

### Table 3
Plating efficiencies (±SEM) for HCT 116 cells after continuous exposure for one (18-h) or two (36-h) cell doubling times to IdUrd alone or in combination with 10 μM 5′-AdThd or 10 μM LV

<table>
<thead>
<tr>
<th>[IdUrd] (μM)</th>
<th>IdUrd alone</th>
<th>IdUrd + 10 μM 5′-AdThd</th>
<th>IdUrd + 10 μM LV</th>
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<td>0.647 ± 0.130</td>
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<td>1</td>
<td>0.633 ± 0.100</td>
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<td>0.302 ± 0.040</td>
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<td>36-h continuous exposure</td>
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<td>0.368 ± 0.031</td>
</tr>
<tr>
<td>2</td>
<td>0.219 ± 0.040</td>
<td>0.038 ± 0.017</td>
<td>0.370 ± 0.020</td>
</tr>
<tr>
<td>5</td>
<td>0.100 ± 0.010</td>
<td>0.005 ± 0.002</td>
<td>0.217 ± 0.037</td>
</tr>
<tr>
<td>10</td>
<td>0.043 ± 0.005</td>
<td>0.0009 ± 0.0004</td>
<td>0.045 ± 0.016</td>
</tr>
</tbody>
</table>

*Control plating efficiency averaged 0.705 ± 0.069.
Fig. 2  A, IdUTP intracellular pool levels in HT 29 cells as a function of IdUrd concentration after 1-h exposure to IdUrd alone (○), or in combination with 10 μM S'-AdThd (■) or 10 μM LV (▲). B, dTTP intracellular pool levels in HT 29 cells as a function of IdUrd after 1-h exposure to IdUrd alone (○), or in combination with 10 μM S'-AdThd (■) or 10 μM LV (▲). Error bars, SE for n ≥ 2 determinations, each done in duplicate.

Fig. 3 Percentage of dThd replacement by IdUrd, in combination with 10 μM S'-AdThd, in HT 29 cells as a function of [IdUrd] for 24-h continuous exposure (A) and 48-h continuous exposure (B). ○, incorporation after exposure to IdUrd alone; □, exposure to the combination of IdUrd with 10 μM S'-AdThd. Error bars, SE for n ≥ 2 determinations, each done in duplicate.

Fig. 4 Percentage of dThd replacement by IdUrd, in combination with 10 μM LV, in HT 29 cells as a function of [IdUrd] for 24-h continuous exposure (A) and 48-h continuous exposure (B). ○, incorporation after exposure to IdUrd alone; □, exposure to the combination of IdUrd with 10 μM LV. Error bars, SE for n ≥ 2 determinations, each done in duplicate.
Fig. 5  A, IdUTP intracellular pool levels in HCT 116 cells as a function of IdUrd concentration after 1-h exposure to IdUrd alone (●), or in combination with 10 μM 5'-AdThd (■) or 10 μM LV (▲). B, dTTP intracellular pool levels in HCT 116 cells as a function of [IdUrd] after 1-h exposure to IdUrd alone (●), or in combination with 10 μM 5'-AdThd (■) or 10 μM LV (▲). Error bars, SE for n ≥2 determinations, each done in duplicate.

Fig. 6  Percentage of dThd replacement by IdUrd, in combination with 10 μM 5'-AdThd, in HCT cells as a function of [IdUrd] for 18-h continuous exposure (A) and 36-h continuous exposure (B). ●, incorporation after exposure to IdUrd alone; ○, exposure to the combination of IdUrd and 10 μM 5'-AdThd. Error bars, SE for n ≥2 determinations, each done in duplicate.

Fig. 7  Percentage of dThd replacement by IdUrd, in combination with 10 μM LV, in HCT 116 cells as a function of [IdUrd] for 18-h continuous exposure (A) and 36-h continuous exposure (B). ▲, incorporation after exposure to IdUrd alone; △, exposure to the combination of IdUrd and 10 μM LV. Error bars, SE for n ≥2 determinations, each done in duplicate.
The effects of 5′-AdThd or LV on the relationship between radiosensitivity and percentage of dThd replacement in HT 29 and HCT 116 cells are presented in Figs. 8 and 9, respectively. In previous studies, we expressed radiosensitization in terms of a change in a (the initial slope of a LQ fit for radiation survival; Ref. 36) because of its usefulness in relating results in vitro to what might be achieved in the clinic, as this parameter reflects changes in the clinically relevant portion of the dose-response curve (radiation doses of 100–300 cGy; Refs. 35 and 36). In Fig. 8A, the slopes of α versus percentage of dThd replacement in HT 29 cells administered alone and in combination with 5′-AdThd were compared and did not differ at one or two cell cycles of exposure (P ≥ 0.12), i.e., the linear relationship of α versus percentage of dThd replacement by IdUrd was not altered by coadministration of 5′-AdThd in HT 29 cells. In Fig. 8B, α was increased significantly at both 24- and 48-hour exposure when 0.5, 1, and 2 μM 5′-AdThd were present (P < 0.007). However, no difference in the slope of α versus percentage of dThd replacement was observed for one and two cells cycles of cotreatment (P > 0.45). Thus, we made the general conclusion that the linear relationship of α versus percentage of dThd replacement by IdUrd was not substantially altered by coadministration of 5′-AdThd and LV in HT 29 cells.

HCT 116 cells are more sensitive to radiation than the HT 29; however, IdUrd is still a very effective in vitro sensitizing agent in these cells (35). In Fig. 9, one and two cell cycles of IdUrd administered alone renders similar linear relationships for α versus percentage of dThd replacement, as reported previously (35). In the presence of 10 μM 5′-AdThd, however, a simple linear fit does not accurately describe the data. When IdUrd has been coadministered with 5′-AdThd, the relationship of α versus dThd replacement appears to become nonlinear around a value of 25%. Any added cytotoxicity due to the combination of IdUrd and 5′-AdThd was controlled for in terms of plating efficiency in these determinations, and radiation experiments at two cell cycles of exposure did not include 5 or 10 μM IdUrd in combination with 10 μM 5′-AdThd because of the excessive toxicity observed (Table 3). Since the addition of 10 μM LV did not affect the percentage of dThd replacement in HCT cells compared to IdUrd alone (Fig. 7), the radiation survival studies were not performed.

**DISCUSSION**

Differences in modulating effects of 5′-AdThd and LV on the extent of DNA incorporation and the toxicity of IdUrd in the HT 29 and HCT 116 colon cancer cells may reflect heterogeneity in the metabolic enzyme profiles of cells from different tumors. For instance, TS activity can vary widely in colon cancer tissues and may be of prognostic value in determining the extent of DNA incorporation and the toxicity of IdUrd in the HT 29 and HCT 116 colon cancer cells. Moreover, TS activity can vary widely in colon cancer tissues and may be of prognostic value in determining the extent of DNA incorporation and the toxicity of IdUrd in the HT 29 and HCT 116 colon cancer cells. Variability in the pattern and extent of dThd replacement has also been noted in experimental studies similar to this study which included modulation of IdUrd metabolism by BrdUrd in human gliomas and in other human colon cancer cell lines (26, 39, 40). In addition, our experiments confirm that dTTP pools are decreased by the addition of IdUrd, as we observed for BrdUrd in another study (40). In the latter case, an increase in intracellular BrdUTP pools was accompanied by a decrease in both the dTTP and dCTP pools, presumably by affecting the equilibrium of metabolism of nucleotides through the ribonucleotide reductase pathway as...
well as through potential inhibitory effects on TS. The build-up of intracellular IdUTP levels observed in our studies is expected to have a similar effect.

On the basis of our results, the potential benefits of these agents as modulators should be assessed more thoroughly in vivo. Our results suggest that the activation of IdUrd to the triphosphate is a major limiting factor in the extent of IdUrd DNA incorporation in two different types of colon cancer cells, and that 5'-AdThd can improve IdUrd DNA incorporation by increasing IdUrd pool levels in both cell lines studied. In addition, 5'-AdThd may have a more selective effect on tumor versus normal tissues, as other in vitro studies have shown that 5'-AdThd did not increase the phosphorylation of thymidine in normal urothelial cells as it did in bladder cancer cells (20-23).

No data have been reported to date for normal gut mucosa and normal bone marrow which are the major dose-limiting normal tissues noted in clinical trials of prolonged continuous infusions of IdUrd (14, 17).

LV is used quite frequently in the clinic as a modulator of the fluoropyrimidines, and the pharmacokinetic parameters of this drug are well characterized (41, 42). In the HCT 116 cells, the data obtained with IdUrd in combination with LV cannot determine whether the levels of drug used in these experiments did not significantly enhance inhibition of TS by IdUMP or whether the competition of dTTP produced de novo was not a significant factor in these cells. In HCT 116 cells, the small increase in IdUrd pools observed when 10 \( \mu \)M LV was coadministered with IdUrd suggests that LV may prevent the breakdown of IdUMP. In contrast, in HT 29 cells, the “lack” of an effect for 10 \( \mu \)M LV on IdUrd levels suggests that the dehalogenation of IdUMP by TS may not be an important factor in this cell line. Additionally, a recent in vivo study demonstrated that coadministration of IdUrd and LV did not increase IdUrd-DNA incorporation in VX2 tumors in rabbits (44).

The levels of modulators used in these experiments are achievable in vivo (30, 41-43), and the percentage of dThd replacement values are in the same range as that observed in the clinic for IdUrd administered as a single agent. The results of this study are most similar to those of the clinic in the case of head and neck tumors, which range from 2.9 to 26.3% dThd replacement, with 63-85% of the cells labeled (4). At these levels in vitro, the relationship of the percentage of dThd replacement and radiosensitization is primarily linear for IdUrd alone or coadministered with either 10 \( \mu \)M 5'-AdThd or LV. However, the effects which radiation dose fractionation or other systemic conditions encountered in vivo may have on the modulation of IdUrd DNA incorporation and radiosensitization by these agents remains to be determined.

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Effects of 5'-aminothymidine and leucovorin on radiosensitization by iododeoxyuridine in human colon cancer cells

EM Miller, KA Kunugi and TJ Kinsella


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