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CELLULAR PHARMACOLOGY OF 5-FLUOROURACIL IN A HUMAN COLON ADENOCARCINOMA CELL LINE SELECTED FOR THYMIDINE KINASE DEFICIENCY

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Abstract—A human colon adenocarcinoma cell line (GC_3TK^-) was selected for thymidine kinase (TK) deficiency from cloned parental cells (GC_3C_1) by exposure to 5-bromodeoxyuridine (BrdUrd). The cellular pharmacology of 5-fluorouracil (FUra) and the influence of physiological concentrations of thymidine (dThd; 0.1 to 1 μM) on FUra cytotoxicity during brief exposure in both cell lines were examined. The uptake of FUra during a 1-hr drug exposure, its metabolism to ribo- and deoxyribonucleotides, incorporation into RNA, and inhibition of thymidylate synthase were similar in GC_3C_1 and GC_3TK^- cells as were the IC_{50} values for FUra (26 and 23 μM respectively). TK deficiency did not reduce the intracellular concentrations of FdUMP generated from FUra. In GC_3C_1, at FUra concentrations up to 100 μM, cytotoxicity was prevented by co-administration of dThd (0.1 to 20 μM). The relationship between cell survival and thymidylate synthase inhibition was close under these conditions. At higher drug concentrations, less dThd protection was observed, and none was detected in GC_3TK^- cells. Thus, the metabolism of FUra did not appear to be altered substantially in GC_3C_1 cells selected for TK deficiency. Also in these cells, at concentrations of FUra <100 μM, FUra cytotoxicity appeared to be mediated via the inhibition of thymidylate synthase.

The mechanism of action of 5-fluorouracil (FUra) in neoplastic cells has been studied extensively. In human colon adenocarcinoma xenografts maintained under the *in situ* conditions of tumor growth, the activity of 5-fluoropyrimidines has correlated with the inhibition of thymidylate synthase [1, 2]. A factor that may influence tumor sensitivity to FUra, however, is the ability of cells to salvage preformed thymidine (dThd), which may circumvent the antithymidylate effect of the drug [3–5].

In humans, serum or plasma concentrations of dThd are slightly lower (0.04 to 0.76 μM; [6–8]) than in the mouse (0.5 to 1.9 μM; [9, 10]). However, it is not yet clear whether these concentrations are adequate to protect cells from FUra cytotoxicity. Previously, we reported the derivation of a thymidine kinase deficient (TK^-) line from GC_3/M human colon adenocarcinoma cells using 5-bromodeoxyuridine (BrdUrd). We considered that this may be a valuable tool for assessing the influence of dThd on FUra cytotoxicity *in vivo*, due to the inability of cells to use the dThd salvage pathway [11]. However, since this line was not originally selected from a clonal derivative of the parent line, there was a possibility that the mutant selected may have additional cellular changes not specifically associated with BrdUrd resistance. Ideally, it would be advantageous to select clones (TK^+, TK^-) which, in the absence of dThd, demonstrated similar survival curves and metabolism of FUra, but where the TK^+ clone was rescued by dThd. Here, we describe the characterization of a TK^- mutant selected from cloned GC_3/M cells (GC_3C_1) that demonstrates these characteristics. The effect of deletion of TK on the cellular pharmacology of FUra, the inhibition of thymidylate synthase, and the definition of the influence of physiological concentrations of dThd on FUra cytotoxicity are reported.

MATERIALS AND METHODS

[5-^{3}H]dUMP (sp. act. 22 Ci/mmol), [6-^{3}H]FdUMP (sp. act. 20 Ci/mmol), [6-^{3}H]FUra (sp. act. 25 Ci/mmol) and [5-*methyl*-^{3}H]dThd (60 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Hanks' balanced salt solution (HBSS) and dialyzed fetal bovine serum (DFBS) were obtained from Gibco (NY); RPMI 1640 medium was from Whittaker Bioproducts (Walkersville, MD), and crystal violet from Accurate Chem (Westbury, NY). Freon and tri-*n*-octylamine were purchased from Aldrich (Milwaukee, WI), and Partisil SAX 10/25 columns from Whatman, Inc. (Clifton, NJ). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or were of reagent grade. The medium was RPMI 1640 containing 10% DFBS and sufficient $CaCl_2$ to bring the total Ca^{2+} concentration (712 μM) to that in RPMI 1640 containing undialyzed FBS, to allow optimal growth. RPMI 1640 medium contained 2.3 μM folic acid such that experiments were conducted under folate-replete conditions. Cells were plated 24 hr prior to conducting experiments unless otherwise stated.

Cell lines. A human colon adenocarcinoma xenograft line ($HxGC_3$) was established previously in continuous culture (GC_3/M; [12]). This cell line was subsequently cloned, and one clone (GC_3C_1) was

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utilized further. TK^- clones were selected by serial exposure of GC_3C_1 to increasing concentrations of BrdUrd from 5 to 50 μg/mL, similar to the procedure described previously [11]. Three hundred cells surviving the highest BrdUrd concentration were subsequently plated in 3 × 100 mm dishes and fed with RPMI 1640 medium containing 10% FBS and BrdUrd (100 μg/mL) for 13 days. Colonies were isolated with glass cloning cylinders and moved to individual wells of a 24-well plate. One clone, $GC_3C_1TK^-C_3$ (GC_3TK^-), was subsequently expanded and characterized. Both parent and drug-resistant lines grew as monolayer cultures.

Growth in selective (HAT) and non-selective medium. GC_3C_1 and GC_3TK^- cells were plated in triplicate at a density of 2×10^5 cells per well in 6-well plates in 3 mL medium. After 24 hr, the medium was replaced either with or without HAT (1 μM aminopterin, 100 μM hypoxanthine, 16 μM dThd). Nuclei were enumerated on day 1 or at 4 and 7 days after incubation at 37°, using a Coulter ZB counter (Hialeah, FL), after cell lysis using the procedure of Butler [13].

Enzyme assays. Thymidine kinase, dThd phosphorylase and thymidylate synthase activities were determined in cultured cells 4 days after plating at a density of 5×10^6 cells in T-175 flasks. For the determination of TK activity, cells were suspended in TNAM buffer (20 mM Tris–HCl, pH 7.4, containing 100 mM NaF, 5 mM AMP and 1 mM β-mercaptoethanol) and homogenized using a polytron (2 × 15 sec; 2°). After centrifugation at 15,500 *g*, 15 min, 2°, cytosol was incubated with 200 μM (5-*methyl*-^{3}H]dThd (sp. act. 2.3 Ci/mmol), 8 mM KCL, 6.7 mM $MgCl_2$ and 5 mM ATP in TNAM buffer, 37°. At 0, 5 and 10 min, 20-μL aliquots were added to 6 μL of ice-cold perchloric acid (PCA, 1 M). After a further 5 min, 2°, 5.3 μL of 2 M KOH was added, and mixtures were centrifuged (15,500 *g*, 5 min, 2°). A 25-μL supernatant fraction was diluted with HPLC buffer (20 mM KH_2PO_4/K_2HPO_4, pH 6.8), and 25 μL of the mixture was analyzed subsequently by HPLC for dTMP, Thy and dThd concentrations, as previously described [11].

For determination of dThd phosphorylase activity, cytosols were prepared in 100 mM KH_2PO_4/K_2HPO_4, pH 8.0, containing 5 mM EDTA and 5 mM β-mercaptoethanol, and were incubated at 37° in the presence of 1.5 mM [5-*methyl*-^{3}H]dThd (sp. act. 30.3 mCi/mmol). Duplicate aliquots of 20 μL were obtained at times, 0, 5 and 10 min, and were processed as described for the assay of TK.

Thymidylate synthase activity was measured in cytosols as previously described [14], after removal of endogenous dUMP by Sephadex G-25 filtration [15]. The level of enzyme was also determined by titration with [6-^{3}H]FdUMP (100 nM) in the presence of 100 μM [6*RS*]CH_2-$H_4PteGlu_1$ as previously reported [16].

FUra cytotoxicity after 1 hr of drug exposure. Cells were plated in triplicate at a density of 3000 cells per well in 6-well plates. After 24 hr, 37°, in an atmosphere of 5% CO_2/95% air, medium was replaced with 3 mL of fresh medium containing FUra at concentrations ranging from 0.1 to 100 μM. After a 1-hr drug exposure, cells were washed with 3 × 3 mL HBSS, and cells were incubated at 37° in medium for a further 7 days. Colonies were fixed in 1 mL of 10% formaldehyde (5 min) and were stained with 1 mL of 0.1% crystal violet followed by washing twice with 0.9% saline. Colonies were enumerated using an automated Artek model 880 colony counter.

Uptake of FUra. GC_3C_1 and GC_3TK^- cells were plated, in duplicate, at densities of 2×10^6 cells per well in 6-well plates. After 24 hr, 37°, cells were exposed to [6-^{3}H]FUra (sp. act. 164 mCi/mmol), at concentrations of 12.5 μM, 25 μM (IC_{50}) and 50 μM. At various times for up to 60 min, drug-containing medium was removed and cells were washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), followed by rapid treatment with 1 mL trypsin (0.05%), EDTA (0.53 mM) and resuspension in 1 ml of ice-cold PBS. Cells were counted, and a 0.75-mL suspension was acidified on ice with 164 μL PCA (2 M). After centrifugation at 15,500 *g*, 2°, 5 min, supernatant fractions were retained, and pellets were re-extracted with 1 mL PCA (0.2 M). Washings and initial supernatant fractions were pooled. Radioactivity was determined in 1-mL fractions after the addition of 10 mL ACS scintillant.

Incorporation of FUra into RNA. Acid-insoluble fractions derived above were mixed with 0.75 mL KOH (1 M) and incubated overnight at 37°. Ice-cold PCA (2 M; 0.375 mL) was added, followed by centrifugation. Radioactivity associated with the RNA fraction was determined in 1 mL of the supernatant fractions, as described above.

Metabolism of FUra to nucleotides. GC_3C_1 and GC_3TK^- lines, plated at a density of 5×10^6 cells in T-75 flasks, were exposed to [6-^{3}H]FUra (1.5 Ci/mmol; 25 or 75 μM) the following day for 30 min at 37°. At the end of incubation, and for up to 24 hr uring further incubation at 37° in drug-free medium, cells were removed by brief trypsinization and washed at 2° with 2 × 3 mL PBS. The final pellets were mixed with 0.5 mL trichloroacetic acid (TCA, 0.6 M) for 10 min at 2° followed by centrifugation (15,500 *g*, 10 min, 2°). TCA was removed from supernatant fractions, and concentrations of FUra ribo- and deoxyribonucleotides were determined by HPLC using the method of Pogolotti *et al.* [17].

Inhibition of thymidylate synthase. Cells were seeded at densities of 1.5×10^6 in each well of 6-well plates, and after 24 hr were treated with concentrations of FUra from 12.5 to 125 μM for 1 hr at 37°. Plates were washed with 3 × 3 mL HBSS, and incubated at 37° for a further 72 hr. Cells were harvested, as described, and initial and residual thymidylate synthase activities were determined at various times as described above.

Influence of dThd on FUra cytotoxicity. The influence of dThd on the cytotoxicity of FUra in parent and GC_3TK^- cells was determined by clonogenic assay. Cells were plated in triplicate at a density of 3000 (GC_3C_1) or 6000 (GC_3TK^-) cells per well in 6-well plates either in the absence or in the presence of 0.1 to 20 μM dThd. After 24 hr at 37°, medium was replaced with the same medium containing FUra (3 μM to 1 mM). After a 1-hr exposure, cells were washed twice in medium and were refed with drug-free medium either in the absence or in the presence of dThd (0.1 to 20 μM). Colonies were enumerated after incubation at 37° for a further 7 days.

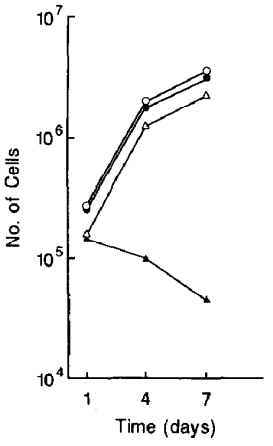

Fig. 1. Growth of GC_3C_1 (○) and GC_3TK^- (△) cells in non-selective medium or in medium containing HAT [(●) GC_3C_1, (▲) GC_3TK^-]. Experimental details are described in Materials and Methods. Data are the means of triplicate determinations. SDs were <14% of the mean.

RESULTS

The growth of GC_3C_1 and GC_3TK^- cells in non-selective and selective medium containing HAT are shown in Fig. 1. In non-selective medium, both lines grew at the same rate, with doubling times of 23–24 hr. However, in medium containing HAT, GC_3TK^- cells were unable to proliferate, whereas the parent line grew normally.

Activities of TK and dThd phosphorylase, and also the activity and level of thymidylate synthase as determined by [6-^{3}H]FdUMP binding, were measured in cytosol fractions from both cell lines (Table 1). It is evident that assays for dThd phosphorylase and thymidylate synthase yielded similar data in GC_3C_1 and GC_3TK^-; the only difference was in the activity of TK, which in the TK^- variant was 10% of that determined in the parent clone.

In RPMI 1640 medium containing 10% DFBS and $CaCl_2$ (712 μM), the IC_{50} for a 1-hr exposure to FUra was similar in GC_3C_1 (26 μM) and GC_3TK^- (23 μM) lines (data not shown).

The uptake of FUra, its metabolism to ribo- and deoxyribonucleotides, incorporation into RNA, and inhibition of thymidylate synthase were compared between both cell lines, using concentrations of FUra based upon the IC_{50} value for a 1-hr exposure (25 μM). The uptake of [6-^{3}H]FUra and its incorporation into RNA were examined over a period of 1 hr at concentrations of 12.5, 25 and 50 μM (Fig. 2). [6-^{3}H]FUra uptake was linear during the first 30 min, and was identical in GC_3C_1 and GC_3TK^- cells. At this time, there was a 72–79% increase in drug accumulation for a 4-fold increase in extracellular drug concentration. The incorporation of radiolabeled drug into RNA was linear over the 60-min period examined, and the extent of incorporation was also identical in both lines (Fig. 2). At 60 min, cells exposed to 50 μM [6-^{3}H]FUra incorporated 3-fold more drug into RNA than cells exposed to 12.5 μM.

The metabolism of FUra was examined at two concentrations of [6-^{3}H]FUra, the IC_{50} (25 μM) and $3 \times IC_{50}$ (75 μM), for a 30-min incubation (during which period uptake was linear), and for up to 24 hr after placing cells in drug-free medium (Fig. 3). For both GC_3C_1 and GC_3TK^- cells, the metabolism of FUra was relatively similar. FUMP, FUDP, FUTP and unbound FdUMP, but not FdUDP or FdUTP were detected. At the end of drug exposure, concentrations of FUMP were higher than FUDP and FUTP, while FdUMP levels were lower, at both concentrations of [6-^{3}H]FUra (Fig. 3). During subsequent incubation in drug-free medium, FUMP declined rapidly over 2 hr, smaller changes in the FUDP pool were detected, and at both concentrations of [6-^{3}H]FUra, FUTP levels increased. FdUMP also increased up to 1 hr after incubation, after which time levels declined. Levels of all nucleotides declined between 4 and 24 hr after drug exposure.

Cells were exposed for 1 hr, 37°, to concentrations of FUra ranging from $0.5 \times IC_{50}$ to $5 \times IC_{50}$, and the influence on thymidylate synthase inhibition was examined for up to 72 hr after exposure (Fig. 4). At the end of incubation, the degree of enzyme inhibition was dose-related and ranged from 18% at 12.5 μM FUra to 95% following 125 μM FUra in both cell lines. The correlation between cell survival and thymidylate synthase inhibition is shown in Fig. 5. At concentrations of FUra up to 75 μM, the relationship between these two parameters was close ($r^2 = 0.977$). When the inhibition of thymidylate synthase was examined at the end of a 1-hr exposure of GC_3C_1 cells to FUra (50 μM) either in the absence or in the presence of dThd (20 μM), no difference in the degree of inhibition was detected (Table 2).

The influence of physiological (0.1 μM, 1 μM) and high (20 μM) concentrations of dThd on the cytotoxicity of FUra in parent and TK^- cells during a 1-hr exposure to FUra with continuous exposure to dThd is shown in Fig. 6. Incubation of GC_3C_1 cells

Table 1. Enzyme activities in cytosolic fractions of GC_3C_1 and GC_3TK^- cells

Cell line	TK*	dThd phosphorylase† (pmol/min/10^7 cells)	Thymidylate synthase‡	[6-^{3}H]FdUMP bound§ (fmol/10^7 cells)
GC_3C_1	57.2	1770 ± 639	89.2 ± 8.1	867 ± 96
GC_3TK^-	5.7	1408 ± 484	96.6 ± 8.8	867 ± 286

*–§ Results are the means of * duplicate determinations or the means ± SD of † four, ‡ six, or § three determinations.

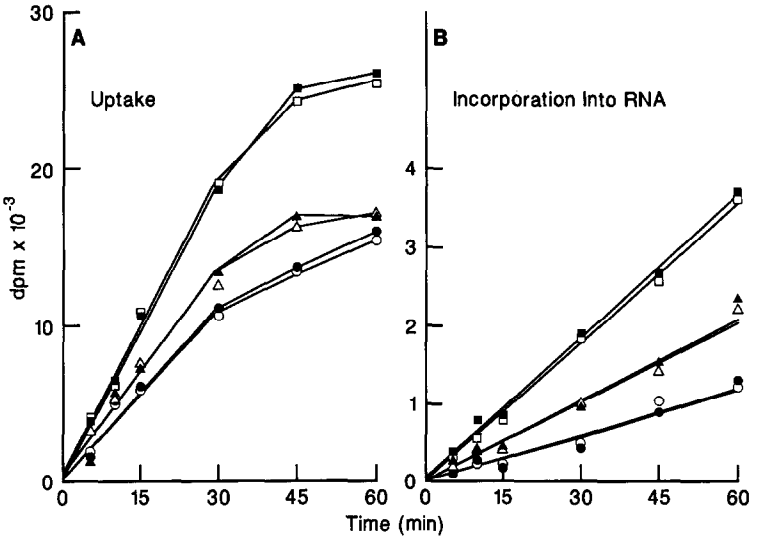

Fig. 2. (A) Uptake of [6-^{3}H]FUra into the acid-soluble fraction and (B) incorporation of [6-^{3}H]FUra into RNA of GC_3C_1 and GC_3TK^- cells after exposure for up to 1 hr to three different concentrations of drug: 12.5 μM (○, ●), 25 μM (△, ▲) and 50 μM (□, ■). Data are the means of duplicate determinations at each time point.

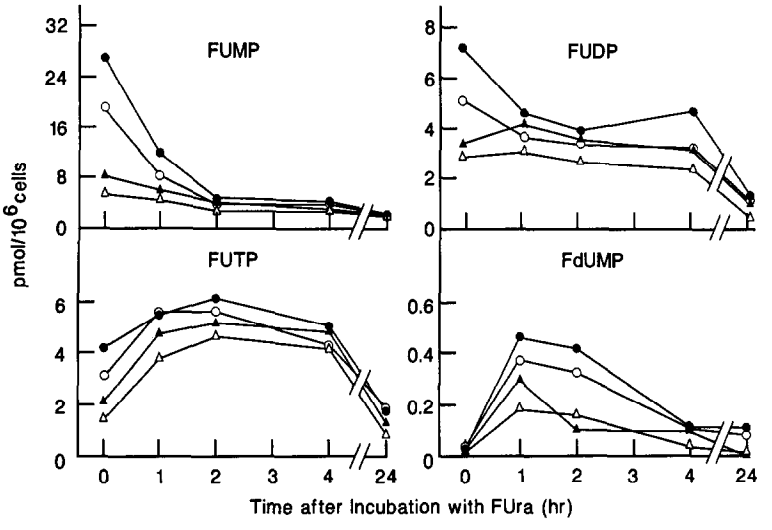

Fig. 3. Metabolism of [6-^{3}H]FUra to FUMP, FUDP, FUTP and FdUMP during and subsequent to a 30-min exposure to [6-^{3}H]FUra at the IC_{50} and 3 × IC_{50} concentrations of FUra in GC_3C_1 (△, ○) and GC_3TK^- (▲, ●) cells respectively. Results are the means of duplicate determinations.

in the presence of FUra and dThd demonstrated an increase in the IC_{50} value for FUra. At all concentrations of dThd, this value was increased by 3.7- to 4.7-fold. Even at a concentration of 100 μM FUra, substantial protection of GC_3C_1 cells was afforded by dThd. However, concurrent administration of dThd had no effect on the cytotoxicity of FUra in GC_3TK^- cells (Fig. 6).

DISCUSSION

Thymidine kinase deficiency in GC_3TK^- cells was confirmed by the inability of these cells to grow in HAT medium, non-reversal of FUra cytotoxicity by dThd, and a 90% reduction in the activity of the enzyme in comparison to wild-type cells. Uptake of [5-^{3}H]Urd, that would be anticipated to be considerably lower in a mutant deficient in dThd transport, was similar in GC_3C_1 and GC_3TK^- cells (unpublished observations). Of interest was that other enzymes that may influence the intracellular levels of thymine nucleotides were similar in both cell types. Some residual TK activity was detected in extracts, but may be due to the presence of mitochondrial TK, which is not deleted by treatment of cells with BrdUrd [18, 19] or, alternatively, may be

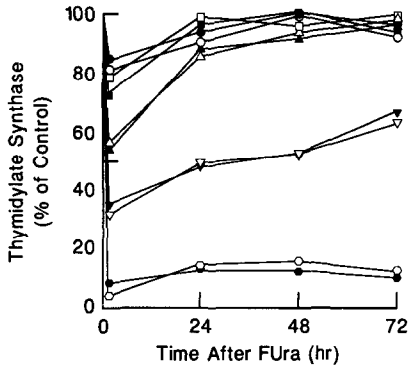

Fig. 4. Inhibition of thymidylate synthase after treatment of GC_3C_1 (open symbols) and GC_3TK^- (closed symbols) cells for 1 hr with FUra at concentrations of 12.5 μM (○, ●), 25 μM (□, ■), 50 μM (△, ▲), 75 μM (▽, ▼) and 125 μM (◇, ◆) respectively. Enzyme activity was determined at the end of drug exposure, and after a further 24, 48, and 72 hr at 37°, as described in Materials and Methods. Each point is the mean of duplicate determinations. Control values: 19,925 dpm/10^6 cells for GC_3C_1 and 18,805 dpm/10^6 cells for GC_3TK^-.

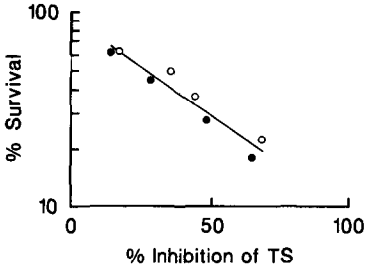

Fig. 5. Relationship between cell survival and thymidylate synthase (TS) inhibition (1 hr after FUra) in GC_3C_1 (○) and GC_3TK^- (●) cells; $r^2 = 0.977$.

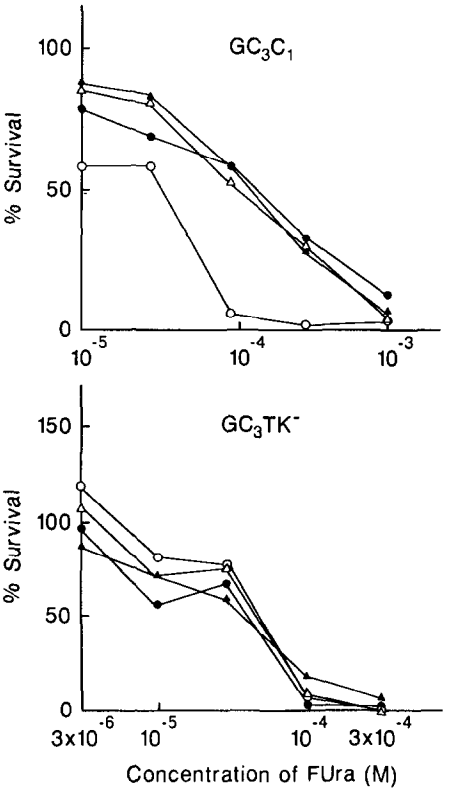

Fig. 6. Influence of dThd on the cytotoxicity of FUra in GC_3C_1 and GC_3TK^- cells after exposure for 1 hr, as determined by the clonogenic assay described in Materials and Methods. Key: (○), 0, (●) 0.1 μM, (△) 1 μM or (▲) 20 μM dThd. Results are the means of triplicate determinations. All standard deviations were within 20% of the mean value, except for 9 of the 40 data points, where the values were within 20–30%.

Table 2. Effect of dThd on the inhibition of thymidylate synthase by FUra in GC_3C_1 cells

Treatment	Thymidylate synthase activity (dpm/10^6 cells)	
None	147,503;	157,795
FUra	43,108;	43,361
FUra + dThd	37,371;	43,108

Dishes (100 mm) were plated, in duplicate, at a density of 3×10^7 GC_3C_1 cells per dish. After 24 hr, cells were incubated with FUra (50 μM) for 1 hr at 37°, either in the absence or in the presence of 20 μM dThd. Following treatment, cells were washed three times in HBSS, harvested, and counted, and thymidylate synthase activity was determined as described in Materials and Methods.

due to incomplete changes in the methylation state of the gene associated with reduced expression of TK [20].

Although the TK^- phenotype was selected from the cloned parent line, there was still the possibility that the metabolism of FUra may be affected in TK^- cells by reduction in the amount of drug metabolized to FdUMP by TK. However, the cytotoxicity of FUra for 1-hr drug exposures, the uptake and incorporation of drug into RNA, and its metabolism to ribonucleotides and FdUMP were very similar, suggesting that TK activity plays an insignificant role in FUra metabolism in these cells. This hypothesis is also substantiated by the observation of a lack of influence of dThd on the inhibition of thymidylate synthase by FUra in GC_3C_1 cells, suggesting no competition between dThd and FdUrd for metabolism to FdUMP in these cells. The highest intracellular FUra nucleotide levels were associated with FUMP, which rapidly declined when cells were placed in drug-free medium, and levels of FUTP and FdUMP became elevated. High levels of FUMP have been detected in L1210 cells following a 22-hr incubation with 0.25 μM [6-^{3}H]FUra [21], although in several mouse and human cell lines [5], and in HCT 116 colon adenocarcinoma cells [22], FUTP appeared to be the predominant FUra metabolite. Data derived on FUra ribonucleotides in the GC_3 cultured cell lines correlated with the higher levels of FUMP determined in $HxGC_3$ xenografts *in vivo*

when the metabolism of [6-^{3}H]FUra was examined (unpublished observations), and thus appear to be indicative of the metabolic characteristics of these cells when grown either *in vitro* or *in vivo*.

Previous studies using gastrointestinal tumor cell lines have shown protection [23] or no protection [23, 24] from FUra cytotoxicity by concurrent administration of dThd. One possibility for protection could be competition between dThd and FdUrd generated from FUra for metabolism by TK. However, that the sensitivities of GC_3C_1 and GC_3TK^- to FUra were similar also indicates that this was not the case, and that rescue occurred by circumvention of thymidylate synthase inhibition. Of note was that the relationship between dose of FUra and degree of thymidylate synthase inhibition for 1-hr drug exposures was similar in both cell lines, and that the correlation ($r^2 = 0.977$) between cell survival and enzyme inhibition was also close for concentrations of FUra up to 75 μM. (Thymidylate synthase inhibition was not examined at a FUra concentration of 100 μM). Further, at concentrations up to 100 μM FUra, cells were substantially protected by dThd, even at physiologically relevant concentrations. Taken together, these data strongly indicate, even after brief periods of drug exposure under conditions that would favor a high frequency of fraudulent base incorporation into RNA, that cytotoxicity is mediated via an antithymidylate or DNA-directed even in these human cells. For 125 μM FUra, however, the correlation between cell survival and thymidylate synthase inhibition was not close, suggesting a shift in the site of FUra cytotoxicity. These data are consistent with the findings of Evans *et al.* [5] in mouse cells, where thymidylate synthase inhibition was the growth-limiting event at low concentrations of FUra (5–20 μM), and RNA-mediated cytotoxicity was growth-limiting at higher concentrations (15–70 μM). For even prolonged exposure to FUra (72 hr), GC_3C_1 cells were protected significantly (1.6- to 3.0-fold) by concentrations of dThd in the range reported in mouse or human plasma, whereas the TK^- variant was not protected by dThd (unpublished observations). Consequently, under conditions where the inhibition of thymidylate synthase by FUra may be prolonged *in vivo*, dThd salvage may play a role in influencing the cytotoxicity of cells to FUra.

It is of interest that cytotoxicity was manifest after relatively transient inhibition of thymidylate synthase. These data suggest that incomplete and relatively short periods of dTTP restriction initiate a chain of events which lead ultimately to cell death. We have reported transient inhibition of thymidylate synthase in tumors following the administration of 5-fluoropyrimidines to tumor-bearing mice [2]. Such inhibition was found in drug-insensitive tumor lines, and we had considered resistance to be a consequence of this. While this conclusion remains valid, the current data obtained *in vitro* indicate that even partial and transient inhibition of thymidylate synthase is associated with cytotoxicity in the absence of dThd salvage. That similar levels and duration of enzyme inhibition may be achieved *in vivo*, without any measurable antitumor effect, suggests that salvage pathways may be important in maintaining cells during this period of decreased synthesis of dTMP *de novo*. We have now isolated clonal lines from GC_3 cells which are similar with respect to metabolism and sensitivity to FUra. As GC_3TK^- is unable to salvage dThd, this will allow definition of the role of dThd salvage *in vivo* to be determined in an unambiguous manner.

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