Fluorodeoxyuridine Modulates Cellular Expression of the DNA Base Excision Repair Enzyme Uracil-DNA Glycosylase

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Abstract

The thymidylate synthase inhibitor 5-fluorouracil (5-FU) continues to play a pivotal role in the treatment of cancer. A downstream event of thymidylate synthase inhibition involves the induction of a self-defeating base excision repair process. With the depletion of TTP pools, there is also an increase in dUMP. Metabolism of dUMP to the triphosphate dUTP results in elevated pools of this atypical precursor for DNA synthesis. Under these conditions, there is a destructive cycle of dUMP incorporation into DNA, removal of uracil by the base excision repair enzyme uracil-DNA glycosylase (UDG), and reincorporation of dUMP during the synthesis phase of DNA repair. The end point is DNA strand breaks and loss of DNA integrity, which contributes to cell death. Evidence presented here indicates that both the nuclear and the mitochondrial isoforms of UDG are modulated by FdUrd (and 5-FU) treatment in certain cell lines but not in others. Modulation occurs at the transcriptional and post-translational levels. Under normal conditions, nUDG protein appears in G1 and is degraded during the S to G2 phase transition. The present study provides evidence that, in certain cell lines, FdUrd mediates an atypical turnover of nUDG. Additional data indicate that, for cell lines that do not down-regulate nUDG, small interfering RNA–mediated knockdown of nUDG significantly increases resistance to the cytotoxic effects of FdUrd. Results from these studies show that nUDG is an additional determinant in FdUrd-mediated cytotoxicity and bolster the notion that the self-defeating base excision repair pathway, instigated by elevated dUTP (FdUTP) pools, contributes to the cytotoxic consequences of 5-FU chemotherapy. (Cancer Res 2006; 66(17): 8829-37)

Introduction

5-Fluorouracil (5-FU) continues to play a pivotal role in the treatment of cancer. This antimitabolite has activity against a wide range of epithelial tumors derived from breast, head and neck, gastrointestinal, and ovarian cancers (1). Several decades of research have provided insights into the mechanism of action of 5-FU. One of its key actions, after activation, is the inhibition of thymidylate synthase. In the cell, 5-FU is converted to fluorodeoxyuridine by thymidine phosphorylase and then to the monophosphate FdUMP by thymidine kinase. Additional pathways function to convert 5-FU to FUMP by reaction with 5-phosphorribosyl-1-pyrophosphate. Phosphorylation to FUDP, reduction by ribonucleotide reductase, and further metabolism of FdUDP also contribute to pools of FdUMP (2). FdUMP in the presence of a reduced folate cofactor forms a stable covalent complex with thymidylate synthase, inhibiting activity and blocking de novo thymidylate (TMP) synthesis (3). The cytotoxic end points of this mechanism include diminished levels of TMP, TTP, and inhibition of DNA synthesis. Other pathways that seem to contribute to the cytotoxic action of 5-FU include extensive incorporation of the ribonucleotide metabolite FUTP into both cytoplasmic and nuclear RNA, disrupting normal RNA homeostasis (4, 5).

Cancer cells vary widely in their sensitivity to this chemotherapeutic agent, and numerous determinants seem to play a role in whether a cell is sensitive or resistant to 5-FU (1). Thus, research efforts continue in an attempt to further elucidate the mechanisms of action of 5-FU in the hope of improving its efficacy in the clinic. Research in several laboratories has discovered a cytotoxic downstream event of thymidylate synthase inhibition that involves a self-defeating base excision repair process (reviewed in ref. 1). Along with the depletion of TTP pools (because of a decrease in de novo TMP synthesis), there is also an increase in dUMP, the normal substrate of thymidylate synthase. Metabolism of dUMP to the triphosphate dUTP results in elevated pools of this atypical precursor for DNA synthesis. In unperturbed circumstances, the enzyme dUTP nucleotidohydrolase (dUTPase) hydrolyzes dUTP to dUMP and prevents its use by DNA polymerase. However, in the presence of 5-FU metabolites, the efficiency of dUTPase seems to be diminished. The result is elevated pools of dUTP (6). Under these conditions, there is a destructive cycle of dUMP incorporation into DNA, removal of uracil by the base excision repair enzyme uracil-DNA glycosylase (UDG), and reincorporation of dUMP during the synthesis phase of DNA repair. The end point is loss of DNA integrity and cell death.

Studies by Camman et al. revealed that levels of dUTPase vary significantly between cell lines. They showed that elevated dUTPase activity in the cell correlated with lower dUTP pools and diminished DNA fragmentation. These investigators also showed that elevated dUTPase correlates with increased resistance to the fluoropyrimidine antimitabolite (7, 8). Introduction of recombinant vectors expressing dUTPase into sensitive cells decreased sensitivity to FdUrd as well as decreasing dUTP pools and DNA fragmentation (9). Using a yeast model system, Tinkelenberg et al. (10) showed that sensitivity to antifolate analogues (aminopterin and sulfanilamide) correlates with dUTPase levels. Increased dUTPase leads to increased resistance, whereas a decrease in dUTPase resulted in greater sensitivity. In addition, this research revealed that inactivation of UDG leads to partial resistance to these antifolate drugs at early times after exposure. More recently, Koehler and Ladner (11) were able to decrease dUTPase levels in cultured cells using small interfering RNA (siRNA) strategies. Suppression of dUTPase using specific

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siRNA resulted in a significant enhancement of dUTP pools, enhanced DNA fragmentation, and a decrease in IC_{50} for FdUrd. Initial studies examining dUTPase expression in paraffin sections by immunohistochemistry suggest that dUTPase expression may be a negative prognostic marker in colorectal carcinomas (12). Additional clinical evidence points to an association between dUTPase levels, survival, and response to 5-FU in colorectal cancer (13). Higher levels of dUTPase protein correlate with poorer response to 5-FU therapy. This study also provides evidence that dUTPase levels vary widely between individual cancer tumor specimens. This variability may be associated with certain mutant forms of p53. Pugacheva et al. (14) determined that certain types of mutant p53s are capable of inducing expression of dUTPase with the result being an increase in resistance of these cells to fluoropyrimidine drugs, although recent work has called into question a direct effect of mutant p53 on dUTPase expression and may be the consequence of indirect effects caused by cancer agent–induced DNA damage and repair (15). In any event, there is clear evidence indicating that elevated levels of dUTPase in cancer cells diminish the effectiveness of the fluoropyrimidine anticancer drugs.

Although considerable data have accumulated on the expression characteristics of dUTPase in several cancer cell lines as well as cancer tissues, very little information exists on the characteristics of UDG expression in cells treated with 5-FU or its metabolites. There are two isoforms of UDG, one located in the nucleus (nUDG, also known as UNG2 or UDG1A) and one that is targeted and processed for mitochondrial DNA repair (mUDG, also known as UNG1 or UDG1; ref. 16). Evidence presented here indicates that both the nuclear and the mitochondrial isoforms of UDG are modulated by FdUrd treatment in certain cell lines but not in others. Modulation occurs at the transcriptional and, at least for nUDG, at a post-translational level. In previous studies, we showed that, with normally cycling cells in culture, nUDG protein expression occurs in the G1 phase of the cell cycle and that, during the S to G2 phase transition, nUDG is degraded by a ubiquitin-mediated process (17). In the present study, we provide evidence that, in certain cell lines, FdUrd mediates an atypical turnover of nUDG in later G1/early S phase of the cell cycle. Additional data indicate that, for cell lines that do not down-regulate nUDG (i.e., HeLa S3), these cells are more sensitive to FdUrd. siRNA-mediated knockdown of nUDG in HeLa S3 cells increases resistance to the cytotoxic effects of FdUrd, increasing the IC_{50} by 6-fold.

Materials and Methods

Cell culture and drug treatments. HeLa S3 cells (CCL-22) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% calf serum. 293H cells were purchased from Invitrogen (Carlsbad, CA); Hs68 (CRL-1635), WE85 (CCL-75), HT29 (HTB-38), CaSki (ATCC CRL-1550), HT-3 (ATCC HTB-32), and SW620 (CCL-227) were purchased from ATCC; and A2780 cells were purchased from Sigma (93112519). Cells were cultured in DMEM (Gibco) with 10% fetal bovine serum and were maintained in a humidified atmosphere of 5% CO_{2} at 37 °C. For Western blot analysis of UDG protein, cells were seeded at 2 × 10^6 per 10-cm dish (except for HeLa and WE85, which were seeded at 1 × 10^6 per dish) 24 hours before FdUrd treatment. Cell extracts were prepared as described previously in extraction buffer containing 50 mmol/L Tris-Cl (pH 7.5), 0.25 mol/L NaCl, 0.1% NP-40, 5 mmol/L EDTA, and 50 mmol/L NaF (17). All cell extraction buffers contained protease inhibitors and phosphatase inhibitors purchased from Calbiochem (San Diego, CA) and 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay system as directed. Where indicated, A2780 cells were treated with nocodazole at a final concentration of 0.4 μg/mL or aphidicolin at 5 μg/mL for the indicated times.

siRNA. siRNAs were supplied by Qiagen Corp. (Valencia, CA). The target sequence specific for the nuclear isoform (nUDG) is ATCCGCGAGA-GACGCCCTCT and starts at the second codon of the open reading frame. Nonsilencing control siRNA was provided by Qiagen and corresponds to the sequence AAATTCTCGAAGCTGTCACGT. Transfections were done using LipofectAMINE 2000 according to the manufacturer’s instructions (Invitrogen). Final concentrations of siRNA used in all experiments were 33 nmol/L.

Western blot analysis. nUDG was identified using ab112 polyclonal antibody specific to the NH_{2} terminus of nUDG (18). This antibody was used at a 1:2,000 dilution for Western blotting. Proteins were resolved under denaturing and reducing conditions on a 12% polyacrylamide gel and transferred to nitrocellulose. Incubation with primary antibody was done from 1 hour to overnight in the same solution. Protein detection was done using goat anti-rabbit IgG conjugated to horseradish peroxidase as secondary antibody and the Luminol system as directed by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, Western blots were stripped and reprobed with antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is indicated in the figures as loading control. To examine both isoforms of human UDG, antibody to the common region of nUDG and mUDG was used (antibody C7). This antibody is relatively weak as an immunostaining antibody using crude extracts. Therefore, UDG was effectively concentrated using Ugi-Sepharose affinity pull-downs from individual cell extracts. These pull-downs were then used for Western blot analysis of both isoforms. These procedures as well as the reagents used have been detailed previously (19).

Mouse monoclonal antibodies (mAb) to thymidine kinase (α988) were purchased from Abcam, Inc. (Cambridge, MA) and used at 10 μg/mL, and mAbs to thymidylate synthase were purchased from Zymed Laboratories (Carlsbad, CA; a subsidiary of Invitrogen) and used at 2 μg/mL.

Flow cytometry. Cells were harvested with trypsin-EDTA and fixed in 70% ethanol. Cells were then incubated with 0.1 mg/mL RNase A and 0.05 mg/mL propidium iodide at room temperature for at least 1 hour. Analysis of DNA content was done using a Beckman Coulter (Fullerton, CA) Epics XL-MCL and Multicycle software.

RNase protection assays. Total RNA was extracted from HT29, 293, Hela, and HS68 cells using Trizol reagent (Invitrogen). mRNA was subsequently isolated using the Oligotex mRNA Midi kit (Qiagen). (α^{32}P)UTP-labeled (Amersham, Piscataway, NJ) antisense RNA probes for UDG and β-actin were synthesized using the Riboprobe In vitro Transcription System (Promega, Madison, WI) as described previously (20). The UDG probe encompasses bases 710 to 840 of exon 1A and protects a 129-bp fragment corresponding to nUDG and a 107-bp fragment corresponding to mUDG. The β-actin probe protects a 227-bp fragment. RNase protection assays were done as directed in the RPA III kit (Ambion, Austin, TX). mRNA (4 μg) and probe (2 × 10^{5} cpm) were used for each hybridization reaction. Products were separated on a 0.75-mm-thick, 5% denaturing polyacrylamide gel (National Diagnostics, Atlanta, GA).

Luciferase reporter assays. The Nucpro and Fullpro fragments were subcloned into the pGL4.11 reporter vector using the Sm{II} and Xho{I} restriction sites (20). This new vector contains an hPEST protein degradation sequence and is designed to respond quicker and with greater magnitude to transcriptional stimuli.

HT29 cells were seeded at 5 × 10^{4} per well (12-well dish) and transiently transfected using LipofectAMINE 2000 according to the manufacturers' directions. The cells were cotransfected with 1 μg reporter plasmid (firefly luciferase) and 10 ng pCMV-Lac (Renilla luciferase, to correct for variations in transfection efficiency). The DNA/liposome complexes were added to the cells for 3 hours and subsequently removed. Complete medium was added plus or minus 100 nmol/L FdUrd. At times indicated, the cells were harvested and analyzed for firefly and Renilla luciferase activity as detailed in the Dual-Luciferase assay System (Promega). Luciferase activities were recorded using a Turner Designs (Sunnyvale, CA; TD-20/20) luminometer. All assays were done in triplicate.
Clonogenicity studies. Basic clonogenic assays followed published reports (21). HeLa S3 cells were seeded at 2 × 10^4 per 6-cm dish in 5 ml of complete medium. Six hours later, these dishes were transfected with the siRNAs. Sixteen hours after transfection, cells were trypsinized and 6-cm dishes were seeded at 1,000 cells per dish. Eight hours after seeding, cells were treated with varying concentrations of FdUrd. Forty-eight hours after FdUrd treatment, each dish was washed with PBS and complete medium (minus FdUrd) was then added. Clones of cells were allowed to develop for 10 to 12 days and then stained with crystal violet. Clones were counted, and the data were analyzed. Experiments were done in triplicate for each concentration, and the experiments were done at least three independent times. Control dishes of transfected cells were harvested at 48 and 72 hours after transfection for Western blot analysis to determine levels of knockdown of nUDG. Average plating efficiencies for HeLa S3 cells under these conditions is 37%.

Statistical analysis. Data were analyzed using Student’s t test and GraphPad (San Diego, CA) Prism version 4 software. Tests of statistical significance were two sided, with differences significant if P < 0.05. IC_{50} values were calculated using GraphPad software and fitting data to sigmoidal dose-response curves with variable slope. SE for the IC_{50} values is based on the 95% confidence interval derived from the IC_{50} calculations.

Enzyme assays. UDG activity was determined using a DNA cleavage assay as described previously (22). A uracil-containing oligonucleotide was 5’-end radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP. Double-stranded substrate, containing a centrally positioned U:A mispair, was generated by annealing the following oligonucleotides: 5’-GGATAGTGTC-CA/GTCTACTGAAGCC-3’ and 5’-GCTTCGACTACGCAACTATCC-3’. DNA cleavage assays contained 1 μg of protein extract, 0.343 μmol/L radiolabeled substrate, 20 mmol/L Tris, 1 mmol/L EDTA, and 50 mmol/L NaCl in a final reaction volume of 20 μL. All reactions were carried out for 10 minutes at 37°C, after which 15 μL of stop/cleavage buffer (70% formamide, 0.3 mol/L NaOH, 1× Tris-borate EDTA) were added to the reaction to achieve cleavage of the abasic site. Samples were heated at 95°C for 15 minutes and separated on a denaturing 20% polyacrylamide gel run at 250 V for 45 minutes and analyzed by autoradiography. Quantitation of reaction products was done by excising the product oligonucleotide (12-mer) and measuring radioactivity in a liquid scintillation counter. Activity is reported as pmol of product formed per minute per mg of protein extract. All assays were done in triplicate. Conversion of substrate to product did not exceed 20% of total substrate and was a linear function of both protein and time within the variables of the assays.

Results

FdUrd affects steady-state levels of UDG protein in certain cell lines. As stated earlier, uracil incorporation and repeated removal of this base may be an additional determinant of 5-FU toxicity. To further characterize the key enzyme that initiates this basic excision repair pathway, nuclear UDG protein levels were examined in response to the 5-FU metabolite FdUrd. The human fibroblast cell lines WI38 and HS68 were treated with 100 mmol/L FdUrd for various times, and protein expression was examined by Western blot. Interestingly, an increase in nUDG protein levels at 24 hours was followed by a significant decrease at 48 and 72 hours after treatment (Fig. 1A). To expand on this, Western blot analysis was done after treating four transformed cell lines, HT29 and SW620 cells showed a similar expression pattern of nUDG expression (Fig. 1B, top). In comparison, HeLa and 293H cells did not significantly alter the expression of nUDG protein in response to the same treatment with FdUrd (Fig. 1B, bottom). FdUrd concentrations up to 1 μmol/L did not alter these expression profiles. It is known that sensitivity to thymidylate synthase inhibition varies markedly between cell lines. A survey of the National Cancer Institute in vitro anticancer screening database (23) using the COMPARE program (http://dtp.nci.nih.gov/docs/compare/compare_intro.html) reveals that sensitivity to FdUrd varies widely about a GL_{50} mean of 2.3 μmol/L.

![Figure 1](http://dtp.nci.nih.gov/docs/compare/compare_intro.html) Western blot analysis of UDG protein levels as a function of FdUrd treatment. A, normal human fibroblasts WI38 and HS68 were incubated in DMEM containing 100 nmol/L FdUrd for up to 72 hours. The cells were harvested every 24 hours, and protein extracts were analyzed by Western blotting. Bottom, nitrocellulose was then stripped and reprobed with anti-GAPDH antibodies to serve as a loading control. B, transformed human cell lines HT29, SW620, 293, and HeLa S3 were incubated in DMEM containing 100 nmol/L FdUrd for up to 72 hours. After harvesting every 24 hours, extracts were made and immunoblot analysis was done on equal amounts of protein extract. Bottom, blots were stripped and reprobed for GAPDH as a loading control. C, HT29 and HeLa S3 cells were treated with 100 nmol/L FdUrd for up to 72 hours. Ugi-Sepharose was used to affinity purify nUDG and mUDG from 50 μg of total protein extract, and complexes were analyzed by Western blotting. Bottom, cell extracts from HT29 and HeLa S3 cells not treated with drug and harvested at the times indicated. D, CaSki and HT-3 cells were treated with 100 nmol/L FdUrd for the indicated times. E, HT29 cells were treated with 5 μmol/L 5-FU for the indicated times, and extracts were then analyzed for nUDG expression. Total cell extract (30 μg) was applied to each lane that was derived from the fibroblast cell lines WI38 and HS68. Total protein extract (10 μg) derived from all other cell lines is applied to each lane. C, control cells not treated with drug.
For the two cell lines we focus on in this work, HT29 cells have a GI50 value of 0.82 μmol/L, whereas inhibition of HeLa cell growth is reported to be in the IC50 range of 0.007 to 0.076 μmol/L (ref. 24 and results reported here). It is interesting to note that the more FdUrd-resistant HT29 cells exhibit loss of nUDG at 100 nmol/L. FdUrd, whereas the more FdUrd-sensitive HeLa S3 cells maintain equivalent levels of nUDG even at concentrations up to 1 μmol/L. We speculate that loss of UDG in HT29 cells contributes to the relative resistance of these cells to FdUrd. Conversely, HeLa S3 cells, by maintaining elevated levels of UDG in the presence of FdUrd, are rendered more susceptible to this agent.

To examine the two isoforms of UDG as a function of FdUrd exposure, Ugi-Sepharose affinity pull-down and an antibody that recognizes both nuclear and mitochondrial isoforms of UDG were used (19). As seen in Fig. 1C, nUDG protein diminishes to almost undetectable levels in HT29 cells but remains constant in HeLa S3 cells, mUDG protein decreases in both cell lines as a function of FdUrd treatment; however, the decrease is significantly less when compared with the decrease of nUDG seen in the four cell lines of Fig. 1A and B. To insure that nUDG expression is not differentially expressed throughout the time course in untreated cells, HT29 and HeLa S3 cells were left untreated and harvested at 0, 24, 48, and 72 hours after plating. As seen in Fig. 1C (bottom), there is no fluctuation of nUDG in either HT29 or HeLa S3 cells. This rules out proliferation effects as a potential cause of differential nUDG expression in HT29 cells.

HeLa S3 cells contain segments of the human papillomavirus (HPV; ref. 25), HPV-18 genome and 293 cells contain sheared fragments of the human adenovirus type 5 genome (26). It is conceivable that the differences seen with nUDG expression may be due to genetic determinants derived from these viruses. To rule this possibility out, two cell lines were obtained from the ATCC that are either positive or negative for HPV. CaSkI (ATCC CRL-1550) contains genetic material from both HPV-16 as well as HPV-18. HT-3 (ATCC HTB-32) is negative for HPV DNA and RNA. Extracts derived from both of these cervical carcinoma cell lines were examined for nUDG expression in each of a function of FdUrd exposure. nUDG protein diminished (Fig. 1D) in both of these cell lines and showed the same profile to the expression pattern seen with HT29 cells (Fig. 1B and C). Therefore, the differences in expression of nUDG between the cell lines examined do not seem to be due to viral influence.

FdUrd is the downstream metabolite of 5-FU, and it has been established that a fraction of 5-FU is converted to FdUrd. However, other metabolites of 5-FU affect the DNA pathway. To see if the clinically used 5-FU also produces similar effects on nUDG, HT29 cells were exposed to 5 μmol/L 5-FU for various times. As seen in Fig. 1E, there is a similar induction of nUDG at 24 hours and then a decrease of nUDG protein at 48 and 72 hours after 5-FU treatment. This is consistent with what is seen with FdUrd exposure.

UDG activity varies considerably from cell line to cell line. In addition, most studies report total activity, which includes both nuclear and mitochondrial enzyme functions. Because we suggest that certain cells reduce UDG levels as a protective mechanism from self-destructing DNA repair, it would be important to know the relative levels of the UDG isoforms in the cell lines under study. Advantage was taken of antibodies that recognize and will only remove nUDG from protein extracts and Ugi-Sepharose affinity procedures to remove total UDG from cell extracts. Table 1 presents activity profiles and corresponding Western blot analysis, indicating relative levels of nUDG and mUDG in three cell lines used in this study. Activity profiles are derived from 1 μg protein extract. As seen in Table 1, HT29 cells have about twice the total activity of HeLa S3 cells. Hs68 cells have significantly lower total activity. nUDG was removed from 50 μg protein extract using antibody 112, and the extract was reanalyzed for UDG activity. Remaining activity is largely represented by the mUDG isoform (column labeled −nUDG activity). As seen under this column, HT29 cells contain higher mUDG activity levels relative to HeLa S3 cells. In Hs68 cells, mUDG seems to contribute all of the UD activity and is consistent with previous reports, indicating that normal fibroblasts contain very low levels of the nuclear isoform of UDG (18). mUDG was then removed from the nUDG-stripped extracts by the Ugi-Sepharose affinity procedure and reanalyzed for UD activity. As seen in the table (under =nUDG−/−mUDG activity column), remaining activity was at or below the limits of detection under the assay conditions used here. To verify that specific isoforms of UDG were removed, each 50 μg fraction (total and −nUDG fraction) was subjected to Ugi-Sepharose affinity pull-downs and analyzed by Western blot. The bottom panel reveals that the specific isoforms were removed from the individual fractions.

Previous research from our laboratory has shown that nUDG is degraded during the S to G2 phase transition of the cell cycle in all cell lines examined to date (17). This seems to be a normal turnover event of this DNA repair enzyme during each cycle of the cell. To insure that cells were not aberrantly arresting in G2 as a consequence of FdUrd treatment, fluorescence-activated cell sorting (FACS) analysis was done. There was no significant increase in G2-M populations when HT29 or HeLa S3 cells were treated with 100 nmol/L or 1 μmol/L FdUrd (Fig. 2; data not shown). In fact, the percentage of HT29 cells in G2 diminished to zero, whereas in HeLa S3 cells the percentage of G2 cells at 12 hours was zero and increased slightly from 24 to 72 hours after treatment. It is known that fluorouracil activates Chk1 kinase and induces an S-phase arrest (28). Despite the variability seen in cell cycle kinetics between HT29 and HeLa S3 cells, the fact that the percentage of cells in the G2 phase diminish indicates that nUDG degradation is not due to the S to G2 phase transition as is the case in unperturbed cycling cells.

To insure that the observed FdUrd-induced modulation of UDG is not a general phenomenon of cell cycle perturbation, we analyzed protein levels of both thymidylate synthase and thymidine kinase. These results are presented in Fig. 3. As illustrated, thymidylate

### Table 1. Enzyme activity of UDG isoforms

<table>
<thead>
<tr>
<th></th>
<th>Total activity</th>
<th>−nUDG activity</th>
<th>−nUDG/−mUDG activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa S3</td>
<td>36.58 ± 1.63</td>
<td>15.04 ± 1.35</td>
<td>≤3.22</td>
</tr>
<tr>
<td>HT29</td>
<td>62.94 ± 2.50</td>
<td>24.33 ± 1.95</td>
<td>≤3.22</td>
</tr>
<tr>
<td>Hs68</td>
<td>7.34 ± 0.81</td>
<td>7.50 ± 0.66</td>
<td>≤3.22</td>
</tr>
</tbody>
</table>

**Note:** Total protein, −nUDG fraction, −nUDG/−mUDG fraction.
synthase shows the characteristic shift in mobility due to FdUMP binding and ternary complex formation (29). There does not seem to be any modulation of protein levels as a function of time after FdUrd exposure in either HT29 or HeLa S3 cells (Fig. 3, top). Thymidine kinase protein levels seem to be induced slightly as a function of FdUrd exposure in both HT29 and HeLa S3 cells (Fig. 3, middle bottom). Neither of these proteins shows modulation similar to the UDG function.

These data allude to the existence of a novel mechanism for the removal of nUDG during FdUrd-induced stress. This mechanism seems to be distinct from the normal S to G2 induced degradation of nUDG and occurs in certain cell lines but not in others.

Steady-state levels of UDG mRNA are modulated by FdUrd treatment of cultured cells. To investigate whether the response to FdUrd is evident at the UDG message level, RNase protection assays were done using mRNA isolated from a variety of cell lines. A single riboprobe was constructed that hybridizes to both isoforms of UDG and produces two distinct products on digestion with RNase. The probe encompasses bases 710 to 840 of exon 1A and protects a 129-bp fragment corresponding to nUDG and a 107-bp fragment corresponding to mUDG (20). As seen with protein levels in HT29 and Hs68 cells, the nUDG message is markedly induced at 12 hours after FdUrd treatment and decreases over the next 36 hours (Fig. 4A, left). As expected, this induction precedes that which is observed for protein levels. In contrast, the level of nUDG mRNA is not greatly affected by treatment with FdUrd in HeLa S3 and 293 cells (Fig. 4A, right). One interesting observation is that mUDG mRNA seems to be regulated coordinately with nUDG. However, this regulation is not reflected at the protein level because mUDG protein only decreases slightly over time (Fig. 1C). This can best be attributed to fundamental differences in protein stability possibly due to mitochondrial compartmentalization. As mentioned earlier, nUDG is normally degraded by an ubiquitin-dependent mechanism in the late S to G2 phase transition, whereas mUDG is not (17).

Reporter studies were next used to determine if the UDG response to FdUrd occurs at the transcriptional or post-transcriptional level. Although Hs68 cells seem to modulate UDG mRNA
levels most vigorously (in response to FdUrd; Fig. 4A), HT29 cells were chosen as they have a much higher transfection efficiency in our hands. Two UDG promoter constructs were generated for these assays, one containing a 600-bp region, located immediately upstream from exon 1A (referred to as Nucpro) and a second larger construct that contains the 600-bp region, exon 1A and first intron (1.2 kb, referred to as Fullpro). Both constructs were generated in pGL4.11, a promoterless vector that has a PEST sequence engineered within the luciferase gene. This produces a less stable luciferase protein that responds more rapidly to transcriptional signals. A schematic representation of these constructs is presented below each graph in Fig. 4. Cells were transfected as described in Materials and Methods and treated with 100 nmol/L FdUrd for 24, 48, or 72 hours. Untreated cells were also harvested at these time points. Results using pGL4.11-Nucpro (Fig. 4B) show a 2-fold increase in luciferase activity above the untreated control at 24 hours. This activity rapidly decreases as FdUrd treatment continues for 48 and 72 hours, indicating that UDG transcriptional activity is initially being activated and subsequently turned off. It should be noted that the decrease in luciferase activity over time associated with untreated cells is a result of the instability of the luciferase protein. Results from pGL4.11-Fullpro reveal a similar trend in response to FdUrd (Fig. 4C). Interestingly, this construct exhibits a more marked decrease in luciferase activity at 72 hours after treatment, which may be due to additional repressive elements located in downstream regions.

FdUrd-induced nUDG protein turnover occurs in the G1 to S phase transition of the cell cycle and is distinct from the normal turnover of this base excision repair protein. In an effort to bolster the argument that FdUrd exposure induces atypical turnover of nUDG in cells, advantage was taken of chemical inhibitors of the cell cycle. Nocodazole treatment was used to block cells in the G2 phase of the cell cycle (30). A2780 cells were chosen for study because they respond well to nocodazole and, in our hands, show a significant G2 block. In addition, nUDG expression patterns in A2780 cells, as a consequence of FdUrd exposure, follow the same pattern as seen with HT29, SW620, and the fibroblast cell lines (Fig. 5C, lanes 1-3 compared with Fig. 1A and B). This is in spite of the fact that A2780 cells seem not to exhibit the same S-phase delay as seen for HT29 and HeLa S3 cells (Fig. 2) as described earlier. Figure 5 shows typical turnover of nUDG during the transition into G2. As can be seen in Fig. 5A and B, disappearance of nUDG is coincident with cells traversing late S into G2. At 12 to 15 hours after nocodazole treatment, where the vast majority of the cells are in G2, nUDG is barely detectable.

In the next experiment, aphidicolin was used to block cells at the G1/early S boundary. The logic for this experiment is based on the premise that, if cells are prevented from transiting S phase into G2, an atypical mechanism should still instigate turnover of nUDG in response to FdUrd. Figure 5A (lanes 1-3) reveals that nUDG derived from A2780 cells decreases significantly at 48 hours after FdUrd treatment. A 24-hour treatment of A2780 cells with aphidicolin results in stable nUDG and corresponds to the G1-S block (Fig. 5C, lanes 1-3).

Figure 4. FdUrd modulates steady-state UDG mRNA levels, and FdUrd modulation of UDG occurs in part at the transcriptional level. A, RNase protection assays were done using mRNA (4 μg) derived from HT29, Hs68, HeLa, and 293 cells as described in Materials and Methods. The cells were treated with 100 nmol/L FdUrd for 12, 24, or 48 hours as indicated before mRNA isolation. Untreated cells were used as control (C). A single 32P-labeled riboprobe (2 × 105 cpm) was added to each reaction, which, on RNase digestion, generated two distinct fragments corresponding to the nUDG and mUDG isoforms. Identical reactions using a 32P-labeled β-actin probe were done as loading controls. Arrows, position of the nUDG and mUDG fragments. B, 600-bp fragment encompassing the nuclear UDG promoter was cloned into the pGL4.11 reporter vector. This construct (Nucpro), along with an expression control (pCMV-luc), was cotransfected into HT29 cells. The cells were subsequently treated with 100 nmol/L FdUrd for the indicated times, and untreated controls were done simultaneously. Luciferase values were normalized using the pCMV-luc control and are presented as fold increase in activity above pGL4.11 (promoterless vector). Points, average of three independent experiments. Bottom, diagram of the region cloned into pGL4.11. C, 1.2-kb fragment encompassing the nuclear promoter exon 1A and first intron of UDG was cloned into the pGL4.11 reporter vector. This construct (Fullpro) was transfected and analyzed exactly as described above. Bottom, diagram of the region cloned into pGL4.11.
Figure 5. Cell cycle–dependent turnover of nUDG and FdUrd-induced turnover of nUDG protein. A, Western blot analysis of A2780-derived nUDG as a function of a nocodazole-induced G2-M block. This study reveals that nUDG begins to decrease during the transition from mid to late S phase (6 hours after nocodazole treatment). At 15 hours after treatment, nUDG protein levels have diminished to undetectable levels. Numbers, hours after nocodazole treatment. B, FACS analysis of A2780 cells after nocodazole treatment. Cells were analyzed at 3-hour intervals. As seen at 0 hour (no treatment), there is a normal distribution of cells with the majority in the G1 phase. Successful G2-M block is revealed at 15 hours, where ~90% of the cells are in the G2-M phase of the cell cycle. C, Western blot analysis of nUDG showing that FdUrd-induced turnover of nUDG is distinct from cell cycle–dependent turnover. Lane 3, 48-hour treatment of A2780 cells with 100 nmol/L FdUrd results in a decrease of nUDG protein similar to patterns observed for other cell lines (Fig. 1). In contrast, 24-hour treatment of A2780 cells with aphidicolin results in stable nUDG (lane 4), and this corresponds with cells blocked in G1-S (D, middle right). Lane 5, when A2780 cells are treated with 100 nmol/L FdUrd for 24 hours and then treated with aphidicolin for an additional 24 hours, nUDG protein is destabilized. D, FACS analysis of A2780 cells treated with a combination of aphidicolin and/or 100 nmol/L FdUrd.

lane 4 and Fig. 5D, middle right). A2780 cells were then treated with FdUrd for 24 hours at which time aphidicolin was added, and the cells were incubated for an additional 24 hours. As can be seen (Fig. 5C, lane 5 and Fig. 5D, bottom left), nUDG decreases to essentially undetectable levels. The cells are largely in S phase with a minor proportion in G1. However, no detectable cells exist in the G2 phase. These data support the notion that there is an atypical turnover of the nUDG protein as a consequence of FdUrd treatment. The evidence also alludes to the possibility that FdUrd-induced turnover of nUDG represents a novel aspect of a checkpoint response. Interestingly, this response does not exist in all cell types (i.e., HeLa and 293).

siRNA knockdown of HeLa S3 nUDG decreases sensitivity of these cells to the effects of FdUrd. Our studies indicate that there is a significant FdUrd-induced down-regulation of nUDG in some cell lines (e.g., HT29), whereas in others the protein levels remain constant (e.g., HeLa S3). We also find that HeLa cells are significantly more sensitive to FdUrd relative to HT29 cells as
discussed earlier. The question arises about whether elevated nUDG levels contribute to FdUrd sensitivity and, conversely, does down-regulation of this protein lead to a decrease in sensitivity to this anticancer agent. Advantage was taken of siRNA strategies to knockdown nUDG in HeLa S3 cells. Cell survival determined through clonogenic assays was then used to evaluate sensitivity to FdUrd. Results of these experiments are presented in Fig. 6. As seen, knockdown of nUDG leads to a significant increase in resistance to the cytotoxic effects of FdUrd. The IC\textsubscript{50} value increases ~6-fold in cells exhibiting siRNA-mediated knock-down of nUDG relative to cells exposed to nonsilencing siRNA (IC\textsubscript{50} 0.466 ± 0.014 μmol/L versus IC\textsubscript{50} 0.076 ± 0.030 μmol/L, respectively; P < 0.0001).

Discussion

Results from these studies show that nUDG is an additional determinant in FdUrd-mediated cytotoxicity and bolster the notion that the self-defeating base excision repair pathway, instigated by elevated dUTP (FdUTP) pools, contributes to the cytotoxic consequences of 5-FU chemotherapy.

Fluorouracil-based treatment of cancer is a widely used regime despite low response rates. As a first-line treatment for advanced colorectal cancer, response rates on the order of 10% to 15% have been reported (31). Encouragement has come from more recent studies reporting on the combination of 5-FU with newer agents, such as irinotecan and oxaliplatin. These combinations have improved overall response rates in certain cases to 50% (32, 33). Positive information, such as this, calls for deeper understanding of the mechanisms involved in 5-FU cytotoxicity and will be important in establishing more successful methods of clinical treatment.

Current understanding of the mechanisms involved in 5-FU toxicity is attributed to inhibition of thymidylate synthase, incorporation of FdUTP and dUTP into DNA, as well as FUTP incorporation into RNA. Thymidylate synthase inhibition results in TTP depletion and significant elevation in the dUTP and FdUTP pools. Normally, the enzyme dUTPase, which hydrolyzes dUTP to dUMP, prevents significant dUMP incorporation into DNA. However, in the presence of fluorouracil metabolites, this enzyme seems not to be capable of handling hydrolysis effectively. Affinity of dUTPase for dUTP and FdUTP is very similar; however, the maximal rate of hydrolysis of FdUTP seems to be ~5-fold lower relative to dUTP (34). Acting as a competitive inhibitor, FdUTP could effectively tie up the enzyme, allowing dUTP pools to build up. Several studies have shown that dUTPase plays an important role in the sensitivity of a cell to thymidylate synthase inhibition (7–9, 35). Expression of this enzyme has been shown to vary widely in tumor tissue. There is also evidence that increased levels of dUTPase correlate with increased resistance to 5-FU treatment (13). A study, which uses siRNA strategies to decrease dUTPase levels in MCF-7 and SW620, reports a significant increase in the sensitivity of these cells to FdUrd (11).

In contrast to current understanding of dUTPase, relatively little information exists on the role of UDG in this cytotoxic process (36, 37). Data presented here indicate that both the nuclear and the mitochondrial isoforms of UDG are modulated by FdUrd treatment in certain cell lines but not in others. This novel modulation occurs at the transcriptional and, at least for nUDG, at a post-translational level. In previous studies, we showed that, with normally cycling cells in culture, nUDG protein expression occurs in the G1 phase of the cell cycle and that, during the S to G\textsubscript{2} phase transition, nUDG is degraded by a ubiquitin-mediated process (17). We provide evidence that FdUrd mediates an atypical turnover of nUDG in late G\textsubscript{1}/early S phase of the cell cycle. This process is conceivably also mediated by ubiquitin-targeted proteolysis. Additional data indicate that, for cell lines that do not down-regulate nUDG, siRNA-mediated knockdown of nUDG increases resistance to the cytotoxic effects of FdUrd, increasing the IC\textsubscript{50} by 6-fold. Recent work by Andersen et al. (38) concludes that excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts and splenocytes. This is in contrast to the results presented here as well as findings from several other laboratories (9, 11, 39). Comparison of data between these two reports is complicated by several factors. These include species differences, tissue-specific differences, and variable growth fractions of the cell types used by Andersen et al. Further study of the mouse system is required to understand these apparent discrepancies as they relate to an S-phase-specific anticancer agent, such as 5-FU.

From the data presented in this report, one can speculate that, in certain cell lines, damage induced by FdUrd induces a novel pathway, possibly associated with a checkpoint mechanism, which culminates in the down-regulation of nUDG. This mechanism protects these cells from the destructive, cyclic base excision repair process created by elevated pools of dUTP. It is conceivable that cells can tolerate uracil in DNA (especially base paired to adenine) much better than they can tolerate DNA strand breaks. Therefore,
if a cell maintains uracil in DNA until pools of dUTP (and FdUTP) decrease, subsequent DNA repair (when the effects of 5-FU are diminished) will be more fruitful, leading to increased cell viability. Critical information supporting this speculation can be seen in UDG knockout mice. Knockout mice, deficient in both isoforms of UDG (ung⁻/⁻), show an elevated steady-state level of uracil in their DNA but do not exhibit an increased spontaneous mutation rate (40) or any evident pathology up to 12 months of age. Mice beyond 18 months begin to reveal lymphoid irregularities (41).

Conversely, cells that lack this mechanism and maintain elevated levels of nUDG are more sensitive to the effects of cyclic base excision repair and show increased toxicity to fluoropyrimidines.

Strategies directed at stabilizing nUDG in cancer cells [perhaps by using newer agents that target, for inhibition, the proteosome complex (42)] may provide a clinically significant approach for novel drug development that, in tandem with the fluoropyrimidines, increase the use of an old but still widely used anticancer agent.

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Fluorodeoxyuridine Modulates Cellular Expression of the DNA Base Excision Repair Enzyme Uracil-DNA Glycosylase

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