0026-895X/93/060854-04$03.00/0

MOLECULAR PHARMACOLOGY, 43:854-857

Mechanisms of Excision of 5-Fluorouracil by Uracil DNA Glycosylase in Normal Human Cells

DAVID J. MAURO, JON K. DE RIEL, RONALD J. TALLARIDA, and MICHAEL A. SIROVER

Fels Institute for Cancer Research and Molecular Biology and the Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received December 28, 1992; Accepted April 2, 1993

SUMMARY

Recent evidence indicates that 5-fluorouracil (5-FlUra) is incorporated into DNA and is removed by the DNA repair enzyme uracil DNA glycosylase. Synthetic oligonucleotides containing either a single uracil or 5-FlUra residue were constructed to examine the mechanisms by which human cells remove 5-FlUra from DNA. The human uracil DNA glycosylase excised uracil in a manner similar to that observed for the bacterial enzyme. In contrast, a significant difference was observed in their abilities to remove 5-FlUra. In particular, both the bacterial and normal human enzymes displayed 13–17-fold increases in their apparent K_m values but the apparent V_{max} values remained virtually constant. These results demonstrate that normal human cells possess a defined capacity to remove 5-FlUra incorporated into DNA. However, specific kinetic differences may exist that affect their capacity to remove 5-FlUra formed in DNA after treatment with this cancer chemotherapeutic agent.

5-FlUra is used as a cancer chemotherapeutic agent for treatment of a variety of tumors. It is the primary agent used in colon cancer chemotherapy (1) and is also utilized in the treatment of a number of other tumors, including breast and ovarian cancer (2, 3). Classically, the efficacy of treatment with this pyrimidine analogue (4) was based on its ability, as 5′-fluorodeoxyuridine monophosphate, to inhibit thymidylate synthetase (5) and its effect on RNA synthesis when utilized as a ribonucleoside triphosphate (6). However, recent evidence indicates that 5-FlUra is incorporated into DNA (7) by conversion to 5′-fluorodeoxyuridine triphosphate and utilization during DNA replication. Although the presence of 5-FlUra in human DNA may result in miscoding during DNA synthesis (8, 9), the relationship between the therapeutic mechanism of action of 5-FlUra and its incorporation into DNA is at present unclear. In particular, the relationship between 5-FlUra-induced cytotoxicity and mutagenicity and its persistence in DNA has not been explored and could represent a significant factor in determining its efficacy.

The DNA repair enzyme UDG functions to remove uracil in DNA, which may arise either by the mutagenic deamination of preexisiting cytidine residues or by the incorporation of 5′-dUMP during DNA synthesis (reviewed in Ref. 10). Initial experiments demonstrated that this enzyme also excises 5-FlUra from DNA. In *Escherichia coli*, mutations in the dUTPase and UDG genes resulted in a 50-fold increase in the level of 5-FlUra in DNA (11). In human lymphoblasts, UDG removed 5-FlUra from a DNA substrate *in vitro* (12). The excision of 5-FlUra from DNA results in the formation of single-stand breaks (13).

Accordingly, we have initially examined the mechanisms by which human UDG removes this DNA lesion. For this analysis, synthetic oligonucleotides were constructed that contained either a single 5-FlUra or uracil residue. We now report that the normal human UDG easily removed uracil from the oligonucleotide, with kinetics comparable to those observed for its bacterial counterpart. In contrast, the human enzyme and the *E. coli* UDG removed 5-FlUra with great difficulty. Initial experiments identified a 13–17-fold increase in the apparent K_m, although the apparent V_{max} remained relatively constant. This finding documents for the first time a unique kinetic distinction in catalysis between the excision of 5-FlUra and that of uracil by the UDG. Thus, normal nontransformed human cells may have a diminished ability to remove 5-FlUra from DNA, based on a reduction in the initial binding of the UDG to this DNA lesion. These results provide a basis to begin to consider the role of 5-FlUra incorporation and removal from DNA in relation to its therapeutic efficacy.

Experimental Procedures

Preparation of UDG. *E. coli* UDG with a specific activity of 1 unit/μl was purchased from Perkin Elmer Cetus. One unit was defined

This study was funded by a grant to M.A.S. from the National Institutes of Health (CA-29414) and by grants to the Fels Institute for Cancer Research and Molecular Biology from the National Institute of Health (CA-12227) and the American Cancer Society (SIG-6). D.J.M. was supported by a predoctoral fellowship from the National Institutes of Health (T32-CA-09214).

ABBREVIATIONS: 5-FlUra, 5-fluorouracil; UDG, uracil DNA glycosylase.

as that amount of protein that released 1 nmol of uracil from a poly(dU)-containing DNA template into acid-soluble material in 60 min at 37°. Human UDG was prepared from confluent nontransformed GM-6112 and GM-6167 normal human skin fibroblasts obtained from the Human Genetic Cell Repository (Camden, NJ). Cells were cultured as monolayers in a sterile humidified atmosphere of 5% CO_2 in air, at a temperature of 37° (14, 15). Cells were grown in 150-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium that contained 5.5 mM glucose (GIBCO BRL, Life Technologies Inc., Gaithersburg, MD) and was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were grown to confluence and then collected by treatment for 10 min with 0.25% trypsin in Hanks' balanced salt solution. Cells were pelleted by centrifugation at 800 × *g* for 10 min at 4°. Cell-free extracts were prepared by suspending cell pellets in 20 mM Tris·HCl, pH 8.0, 1 mM dithiothreitol, 20% glycerol, and then sonicating the suspension at 60 W for 20 sec on ice, with a Braunsonic needle probe. Cell debris was removed by centrifugation at 2300 × *g* for 10 min at 4°. Protein was determined as described (16).

Determination of UDG activity. Four oligonucleotides (27 bases in length) were prepared using an Applied Biosystems DNA synthesizer. Three of the oligonucleotide strands were composed of identical bases with the exception that one contained a uracil or 5-FlUra, rather than a thymine, at position 16. The third strand was complementary. Full length oligonucleotides were purified through a 20% polyacrylamide/7 M urea gel. The strands were excised and allowed to diffuse into deionized-distilled water. The concentration of each oligonucleotide was determined by calculation of the $A_{260/280}$ ratio. Oligonucleotides were then 5′-end labeled using polynucleotide kinase. The labeling reaction and subsequent purification were performed according to the method of Maniatis *et al.* (17). Thin layer chromatography after the labeling reaction demonstrated that >99% of the oligonucleotides were end labeled (SurePure; United States Biochemicals). Oligonucleotides were then annealed at equimolar concentrations, heated at 65° for 2 min, and then allowed to cool to room temperature. The specific activity of the resulting double-stranded oligomers was 10,000 cpm/pmol. UDG activity was quantitated in a reaction buffer (total volume, 150 μl) that contained 0.1 M Tris·HCl, pH 7.5, 0.01 M K_2EDTA, 5 mM dithiothreitol, and radiolabeled oligonucleotide. The reaction was performed at 37° for 30 min. The mixture was then placed in a Savant speed vacuum until dry. Piperidine (1 M) was added and the mixture was heated for 30 min at 90°. The sample was dried and washed twice with water. Oligonucleotides were separated on a 20% polyacrylamide/7 M urea gel at 700 V for 5 hr. The gel was then exposed to Kodak X-OMAT film.

Densitometric analysis. Densitometry was performed using a Bio-Rad scanning densitometer. Quantitation of band density was determined using a Gaussian fit for curve integration. In each assay, standard curves were created using known standard concentrations loaded on the gel adjacent to the products of the respective enzyme assay. Standard curves were created by comparing the amount of standard used versus density. This was used as an internal reference to determine the relative band density of the respective reaction products in that specific assay.

Results

Because 5-FlUra occurs in DNA only as a result of the use of 5′-fluorodeoxyuridine triphosphate during DNA synthesis, synthetic oligonucleotides were constructed that contained uracil, 5-FlUra, or thymine in a complementary base pair opposite adenine (Fig. 1A). The utility of these synthetic oligonucleotides in the UDG assay was determined first by reaction with the highly purified bacterial enzyme (Fig. 1B). Two radiolabeled bands were observed when the uracil substrate was used and the resulting apyrimidinic site was cleaved chemically by the addition of 1 M piperidine (Fig. 1B, *lane 1*). In contrast, in the absence of the enzyme only the parent oligonucleotide containing a single uracil was detected (Fig. 1B, *lane 2*). Similarly, using an oligonucleotide containing thymine no cleavage was observed in the presence or absence of the bacterial UDG (Fig. 1B, *lanes 3* and *4*, respectively). Accordingly, these results define the specificity of the assay and, because only one low molecular weight band was observed in any of the experiments, demonstrate that oligonucleotide cleavage could not result from a nonspecific nuclease activity.

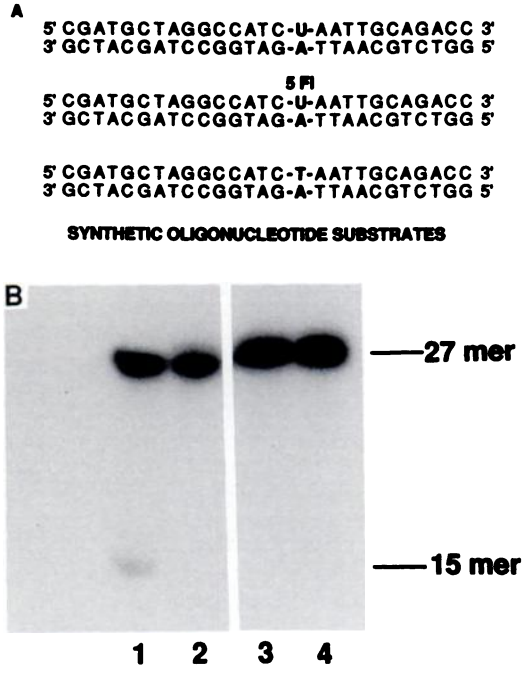

Fig. 1. Specificity of uracil release from 27-mer synthetic oligonucleotides. A, Oligonucleotides containing U/A, 5-FlUra/A, and T/A complementary base pairs were constructed as described in Experimental Procedures. B, UDG activity was examined using 0.1 units of *E. coli* UDG. *Lane 1*, *E. coli* UDG plus uracil-containing oligonucleotide plus 1 M piperidine; *lane 2*, uracil-containing oligonucleotide plus 1 M piperidine; *lane 3*, *E. coli* UDG plus thymine-containing oligonucleotide plus 1 M piperidine; *lane 4*, thymine-containing oligonucleotide plus 1 M piperidine.

The activity of the bacterial UDG with the uracil-containing oligonucleotide was then examined. As defined by the appearance of the 15-mer, enzyme catalysis was determined as a function of protein concentration at different intervals (Fig. 2A). Enzyme activity was then quantitated by densitometry. Little activity was observed with 3.3–6.6 × 10^{-5} μg of the *E. coli* enzyme, in incubations of up to 30 min. Catalysis increased proportionately between 3.3 × 10^{-4} and 3.3 × 10^{-2} μg of protein over this time interval. In contrast, the reaction was complete at 5 min with 0.33 μg of enzyme. Two different nontransformed fibroblast cell strains were used to examine the activity of the normal human UDG. In both GM-6112 and GM-6167 cells, UDG activity was proportional to the time of incubation and to the amount of protein added to the UDG assay (data not shown).

The ability of the human UDG to remove 5-FlUra from DNA was examined using a 27-mer containing a single 5-FlUra at position 16. Production of the 15-mer was dependent on the amount of enzyme and the incubation time (data not shown). Quantitation of the rate of 5-FlUra release revealed that demonstrable release of 5-FlUra was observed using 0.5–5 μg of cell protein. However, the extent of 5-FlUra release by the normal human enzyme was significantly lower than that ob-

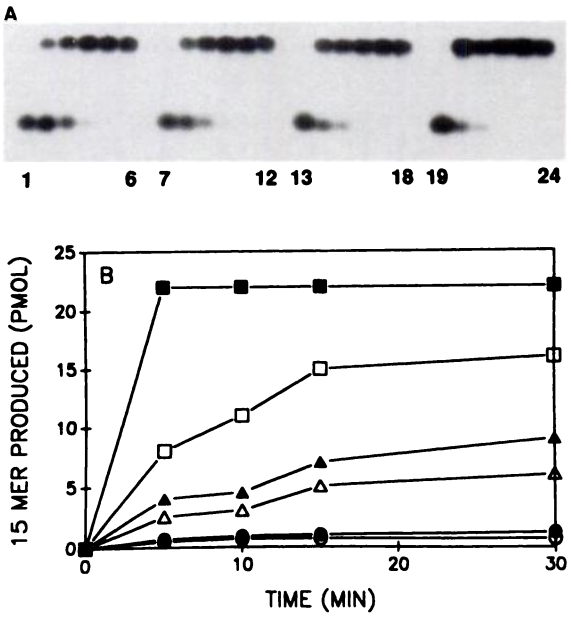

Fig. 2. Determination of *E. coli* UDG activity. *E. coli* UDG was quantitated as described in Experimental Procedures, using 20 pmol of uracil-containing oligonucleotide. A, *Lanes 1-6*, *7-12*, *13-18*, and *19-24*, incubations with decreasing amounts of enzyme assayed for 30, 15, 7.5, and 2.5 min, respectively. B, Densitometry was performed as described. ○, 3.3×10^{-5}; ●, 6.6×10^{-5}; △, 3.3×10^{-4}; ▲, 6×10^{-3}; □, 0.03; ■, 0.33 μg of *E. coli* UDG.

served for uracil. Furthermore, the time interval required was markedly increased. Similar results were observed using the highly purified bacterial enzyme.

Kinetic comparisons were then utilized to consider the molecular mechanism that may underlie the lower rate of 5-FlUra excision observed with both the *E. coli* UDG and the normal human enzyme. As shown in Fig. 3A, the *E. coli* enzyme removed uracil from the 27-mer with a significantly increased initial rate, compared with that observed for 5-FlUra excision. However, as the substrate concentration increased the rate of excision using either substrate diminished in parallel. Similar results were observed with the normal human UDG from either the GM-6112 (Fig. 3B) or GM-6167 (Fig. 3C) cell strain. In both cases, the initial rate of uracil excision at low substrate concentrations was significantly higher than that observed for 5-FlUra. However, at higher concentrations a similar reduction in the excision rate was observed.

Kinetic constants were determined using nonlinear regression analysis (Table 1). The K_m of the bacterial enzyme for uracil release was calculated to be 0.28 ± 0.03 μM, whereas the K_m for 5-FlUra removal was 4.99 ± 1.36 μM. This represented a 17-fold increase in the K_m for 5-FlUra excision, compared with uracil removal. However, quantitation of the V_{max} demonstrated a small decrease, from 0.26 ± 0.01 pmol/min to 0.21 ± 0.03 pmol/min, respectively. A similar result was observed with respect to catalysis by the normal human enzymes. With the uracil substrate the apparent K_m and apparent V_{max} of the GM-6112 enzyme were 0.22 ± 0.05 μM and 0.17 ± 0.01 pmol/min, respectively. These were statisically indistinguishable from the apparent K_m and V_{max} values of 0.34 ± 0.15 μM and 0.19 ± 0.02 pmol/min, respectively, calculated for the GM-6167 UDG. In contrast, with the 5-FlUra substrate an apparent K_m and apparent V_{max} of 3.24 ± 0.97 μM and 0.11 ± 0.01 pmol/min, respectively, were calculated for the GM-6112 UDG. For the GM-6167 UDG the apparent K_m and apparent V_{max} were 3.28 ± 1.14 μM and 0.11 ± 0.02 pmol/min, respectively. Thus, a 13–16-fold increase in the apparent K_m was detected for the removal of 5-FlUra, compared with uracil excision, whereas virtually no change was observed in the apparent V_{max}. These results suggest that a difference in the binding of the UDG to a 5-FlUra substrate underlies the reduced ability of this enzyme from either *E. coli* or normal human cells to remove 5-FlUra from DNA.

Discussion

An understanding of the molecular mechanisms of DNA repair is fundamental to our knowledge of the mechanisms by which we defend ourselves against the initiation of neoplasia after DNA modification and damage. In contrast, tumor cells may utilize the identical pathways to mitigate the DNA-damaging effects of cancer chemotherapeutic agents. In this instance, tumor cell survival is facilitated by the ability of DNA repair mechanisms to remove lesions induced during therapy.

To examine this duality of function, the mechanisms by which normal human cells remove uracil or 5-FlUra from DNA has been investigated using synthetic oligonucleotide templates

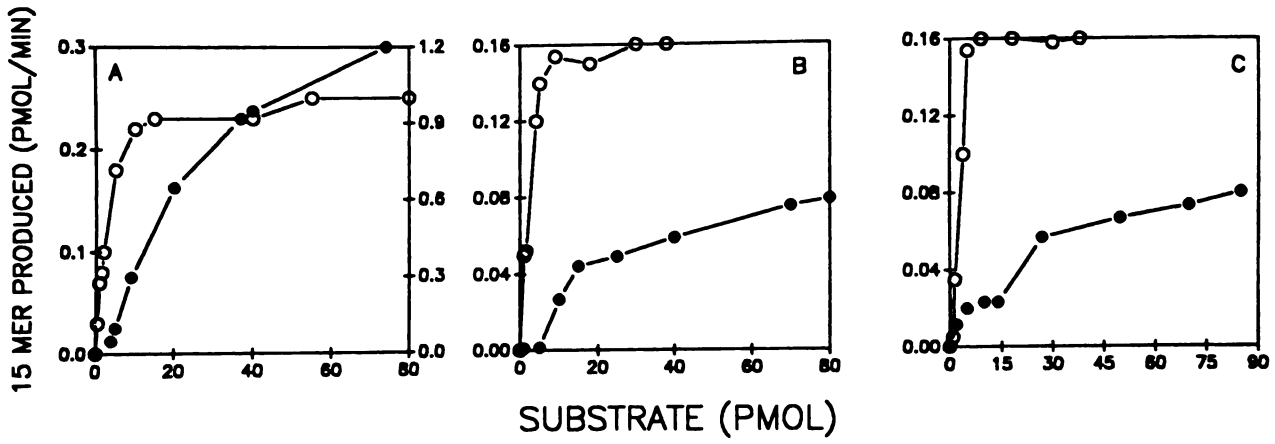

Fig. 3. Comparison of 5-FlUra and uracil excision by the UDG. Kinetic analysis was performed as described in Experimental Procedures. A, *E. coli* UDG; B, normal human GM-6112 UDG; C, normal human GM-6167 UDG. ○, Uracil-containing 27-mer; ●, 5-FlUra-containing 27-mer.

TABLE 1

Kinetic analysis of 5-FlUra excision

Determinations using the crude human protein preparations reflect an apparent K_m and apparent V_{max}.

Source	Substrate (27-mer)	K_m	V_{max}
		μM	pmol/min
E. coli	Uracil	0.28 ± 0.03	0.26 ± 0.01
	5-FlUra	4.99 ± 1.36	0.21 ± 0.03
Human (GM-6112)	Uracil	0.22 ± 0.05	0.17 ± 0.01
	5-FlUra	3.24 ± 0.97	0.11 ± 0.01
Human (GM-6167)	Uracil	0.34 ± 0.15	0.19 ± 0.02
	5-FlUra	3.28 ± 1.14	0.11 ± 0.02

containing either a single uracil or 5-FlUra residue. The results presented in this study demonstrate that the human UDG displayed an increase in the apparent K_m for 5-FlUra excision that was indistinguishable from the increase in the actual K_m observed for the *E. coli* UDG. Similarly, the relative consistency in the apparent V_{max} of the human UDG for uracil or 5-FlUra excision was identical to that determined for the bacterial enzyme.

These investigations identify a potential kinetic mechanism that underlies the reduced activity of the human UDG to remove 5-FlUra from DNA. In particular, determination of the apparent K_m and the apparent V_{max} values using a uracil-containing and a 5-FlUra-containing oligonucleotide demonstrated a 1 order of magnitude difference in the apparent K_m with the 5-FlUra substrate. In contrast, there was virtually no change in the apparent V_{max}, compared with that for the uracil-containing oligonucleotide. The increased apparent K_m would suggest that the initial binding of the human UDG to the 5-FlUra-containing substrate is quite difficult. However, as illustrated by quantitation of the apparent V_{max}, the rate of catalysis by the human UDG is identical with 5-FlUra and uracil once the enzyme-substrate complex has been formed. Further investigations using extensively purified human UDGs may define a potential relationship between such kinetic differences and the efficacy of treatment using this cancer chemotherapeutic agent.

References

1. Carter, S. K. Future directions in the therapy for large bowel cancer. *Cancer (Phila.)* **50**:2647–2656 (1982).
2. Cooper, R. G. Adjuvant chemotherapy for breast cancer: 20 years experience using CVFMP chemotherapy. *Semin. Oncol.* **15**(Suppl. 3):29–34 (1988).
3. Alberts, D. S., D. Garcia-Kendall, and E. A. Surwit. Phase II trial of mitomycin C plus 5-FU in the treatment of drug-refractory ovarian cancer. *Semin. Oncol.* **15**(Suppl. 4):22–26 (1988).
4. Duschinsky, R., E. Pleven, and C. Heidelberger. The synthesis of 5-fluoropyrimidines. *J. Am. Chem. Soc.* **79**:4559–4560 (1957).
5. Hartmann, K.-U., and C. Heidelberger. Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. *J. Biol. Chem.* **236**:3006–3013 (1961).
6. Kufe, D. W., and P. P. Major. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *J. Biol. Chem.* **256**:9802–9805 (1981).
7. Lonn, U., and S. Lonn. Interaction between 5-fluorouracil and DNA of human colon adenocarcinoma. *Cancer Res.* **44**:3414–3418 (1984).
8. Aebersold, P. M. Mutation induction by 5-fluorodeoxyuridine in synchronous Chinese hamster cells. *Cancer Res.* **39**:808–810 (1979).
9. Kremer, A. B., T. Mikita, and G. P. Beardsley. Chemical consequences of incorporation of 5-fluorouracil into DNA as studied by NMR. *Biochemistry* **26**:391–397 (1987).
10. Sirover, M. A. Cell cycle regulation of DNA repair enzymes and pathways, in *Transformation of Human Fibroblasts* (G. P. Milo and B. C. Casto, eds.). CRC Press, Boca Raton, FL, 29–54 (1990).
11. Warner, H. R., and P. A. Rockstroh. Incorporation and excision of 5-fluorouracil from deoxyribonucleic acid in *Escherichia coli*. *J. Bacteriol.* **141**:680–686 (1980).
12. Ingraham, H. A., B. Y. Tseng, and M. Goulian. Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Res.* **40**:998–1001 (1980).
13. Lonn, U., and S. Lonn. DNA lesions in human neoplastic cells and cytotoxicity of 5-fluoropyrimidines. *Cancer Res.* **46**:3866–3870 (1986).
14. Gupta, P. K., and M. A. Sirover. Sequential stimulation of DNA repair and DNA replication in normal human cells. *Mutat. Res.* **72**:273–284 (1980).
15. Gupta, P. K., and M. A. Sirover. Regulation of DNA repair in serum stimulated xeroderma pigmentosum cells. *J. Cell Biol.* **99**:1275–1281 (1984).
16. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

Send reprint requests to: Michael A. Sirover, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140.