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Selective Inhibition of Herpes Simplex Virus Type-1 Uracil-DNA Glycosylase by Designed Substrate Analogs*[§]

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Cytosine deamination and the misincorporation of 2'-dUrd into DNA during replication result in the presence of uracil in DNA. Uracil-DNA glycosylases (UDGs) initiate the excision repair of this aberrant base by catalyzing the hydrolysis of the *N*-glycosidic bond. UDGs are expressed by nearly all known organisms, including some viruses, in which the functional role of the UDG protein remains unresolved. This issue could in principle be addressed by the availability of designed synthetic inhibitors that target the viral UDG without affecting the endogenous human UDG. Here, we report that double-stranded and single-stranded oligonucleotides incorporating either of two dUrd analogs tightly bind and inhibit the activity of herpes simplex virus type-1 (HSV-1) UDG. Both inhibitors are exquisitely specific for the HSV-1 UDG over the human UDG. These inhibitors should prove useful in structural studies aimed at understanding substrate recognition and catalysis by UDGs, as well as in elucidating the biologic role of UDGs in the life cycle of herpesviruses.

Uracil-DNA glycosylases (UDGs)¹ are a highly conserved class of DNA repair enzymes that initiates excision repair of uracil in DNA by hydrolyzing the *N*-glycosidic bond (Fig. 1*a*). Uracil in DNA arises from the misincorporation of deoxyuridine triphosphate (dUTP) during replication and from the hydrolytic deamination of cytosine (1, 2). Cytosine deamination generates highly mutagenic G:U mismatches that lead to G:C to A:T transition mutations. Characterized by a high substrate specificity, UDGs are able to distinguish between such structurally similar bases as uracil and thymine. Unlike other DNA glycosylases, UDGs remove uracil from both single-stranded and double-stranded DNA, often with higher efficiency for the

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¹ The abbreviations used are: UDG, uracil-DNA glycosylase; HSV-1, herpes simplex virus type-1; VZV, varicella-zoster virus; β FdUrd, 2'- β -fluoro-2'-deoxyuridine; fhUrd, furan homouridine; EMSA, electrophoretic mobility shift assay; ds, double-stranded; ss, single-stranded.

single-stranded substrates (1, 3).

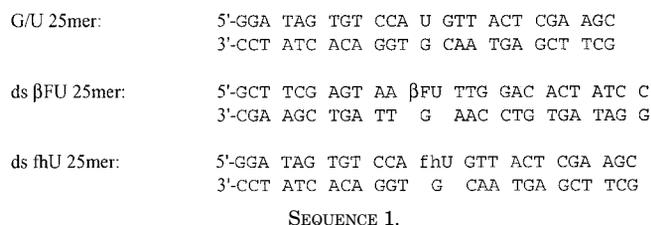
DNA glycosylases accomplish the formidable task of scanning a large excess of normal DNA bases, recognizing and then catalyzing the excision of damaged bases. A molecular-level description of how DNA glycosylases achieve recognition and repair requires the formation of stable protein-substrate complexes that can be studied by x-ray crystallography or NMR. Such studies are not ordinarily possible because of the transient nature of the enzyme-substrate interaction. One way to circumvent this problem involves mutating the enzyme so as to obtain a catalytically inactive form that retains the ability to recognize its substrate (4, 5). An alternative approach is in effect to "mutate" the substrate by preparing synthetic analogs that bind the enzyme specifically but cannot be processed by it (6–8). Both approaches have proven useful in structural and biochemical analysis of base excision DNA repair (4–12). Powerful features of the substrate modification approach are that it requires no prior knowledge of the enzyme active site and that it can be structurally very conservative, entailing changes of as little as a single atom.

UDGs have an unusually broad phylogenetic distribution, being present in organisms from the simplest free-standing bacterium (13) to plants (14) to man (15). Furthermore, UDGs have been identified in the genomes of herpesvirus and poxvirus. HSV-1 UDG is dispensable for HSV-1 replication in cell culture, presumably because the cellular UDG can supplant the activity of the viral enzyme (16, 17). However, an intact HSV-1 UDG gene appears to be necessary for efficient viral replication and reactivation in the murine nervous system (18). The difference in the efficiency of viral replication and reactivation between the *in vitro* and *in vivo* studies has been attributed to the lack of detectable endogenous UDG expression in neurons, which are terminally differentiated (19). Studies of HSV-1 in neural systems are relevant because HSV-1 establishes latency in the neuronal ganglia (20). Despite these results, the role of UDG in herpesvirus replication and reactivation remains unclear. For example, varicella-zoster virus (VZV), another member of the herpesvirus family, has been shown to replicate in the absence of detectable endogenous or host UDG activity (16). Human cytomegalovirus (CMV), another herpesvirus, is unlike HSV-1 or VZV in that the disruption of its UDG gene results in a longer replication cycle with delayed DNA synthesis in tissue culture (21). Potent and highly specific inhibitors of viral UDGs could serve as valuable tools to elucidate the *in vivo* role of the enzyme in herpesvirus replication and virulence. The availability of such molecules could provide the impetus to pursue HSV-1 UDG as a novel target for anti-viral therapy (22).

Despite the potential therapeutic interest in HSV-1 UDGs, few synthetic inhibitors of UDGs have been reported. The most potent non-protein UDG inhibitors thus far reported have IC₅₀ values in the micromolar range (23, 24). Here, we report the design, synthesis, and evaluation of two inhibitors of UDGs. Our results demonstrate that these two oligonucleotide-based inhibitors bind and inhibit HSV-1 UDG while having little effect on the human enzyme.

EXPERIMENTAL PROCEDURES

Enzymes—HSV-1 and human UDG (UDG Δ 84) were generous gifts from Prof. Laurence H. Pearl (Department of Biochemistry and Molecular Biology, University College, London) and Prof. Hans Krokan (UNIGEN Center for Molecular Biology, The Norwegian University of Sci-



ence and Technology), respectively.

Oligonucleotide Substrates/Competitors—All oligonucleotides were synthesized by standard β -cyanoethyl solid phase chemistry on a 1 μ mol scale using an ABI model 392 DNA synthesizer and were purified by denaturing polyacrylamide gel electrophoresis. End-radiolabeling reactions were performed with T4 polynucleotide kinase (New England Biolabs) and γ - 32 P]ATP (PerkinElmer Life Sciences). Duplexes were prepared by annealing with 10-fold excess of complementary strand. The radiolabeled strand of each duplex is the top strand, as shown in Sequence 1. The phosphoramidite of 2'- β -fluoro-2'-deoxyuridine (β FdUrd) was synthesized according to published procedures (25). The phosphoramidite of furan homouridine (fhUrd) was synthesized by an adaptation of a published procedure (26).²

Electrophoretic Mobility Shift Assays (EMSA)—The standard binding reaction mixture (20 μ l) contained varying amounts of protein and 0.4 fmol of 32 P-labeled oligonucleotides in 100 μ M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 50 mM Tris, pH 7.4. After 30 min of incubation at room temperature, the samples were resolved on a 10% native polyacrylamide gel in 0.5 \times Tris-borate-EDTA running buffer. The thermodynamic dissociation constants ($K_d = [\text{protein}][\text{DNA}]/[\text{protein-DNA complex}]$) were measured as the concentration of the protein at which half of the target DNA is bound, under conditions in which $[\text{DNA}] \ll K_d$. Data from at least three titration gels were averaged to obtain the reported K_d values, which have standard errors of \pm 50%. Example gels and binding data used to determine the experimental equilibrium constants are available as supplemental information.

Cleavage Inhibition Assays—The standard reaction mixture (20 μ l) contained 20 fmol of 32 P-labeled dU/G duplex oligonucleotides, varying dilutions of enzyme and 1 pmol of competitor oligonucleotide duplex. The reaction buffers were as follows: human UDG (60 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 8); HSV-1 UDG (20 mM Tris-HCl, 10 mM EDTA, pH 8). The reactions were incubated for 30 min at 37 $^{\circ}$ C for HSV-1 UDG and 10 min at 30 $^{\circ}$ C for human UDG and were stopped by the addition of 10 μ l of formamide loading dye and 5 μ l of 2 M piperidine. The sample mixtures were then boiled for 30 min prior to resolving on a 20% polyacrylamide gel.

RESULTS AND DISCUSSION

Design and Synthesis of UDG Inhibitors—Like all DNA glycosylases, UDG processes its substrate through a transition state having partial dissociative (SN1) character, wherein positive charge is accumulated on the sugar ring, especially at C-1' and O-1' (27–29). Inhibitors have been designed either to mimic this transition state or to destabilize it electronically. The latter principle was employed in this study. Namely, analog **1** (2'- β -fluoro-2'-deoxyuridine, β FdUrd) (Fig. 1c) possesses a 2'-fluoro substituent, which destabilizes the transition state by withdrawing electron density from the already electron-deficient C-1' center. Analog **2** (fhUrd) (Fig. 1c) interposes a CH₂ unit between the base and its sugar, thereby depriving the transition state of assistance from the lone pair electrons on O-1'. The increased distance from C-1' to the base in **2** may resemble the lengthening of the glycosidic bond that occurs during displacement of the base (Fig. 1b). Both analogs possess an attached uracil base, which UDG is expected to recognize specifically. Indeed, analog **1** has previously been shown to bind and inhibit the human G/T glycosylase at subnanomolar concentrations (12).

The β FdUrd and fhUrd nucleoside analogs were synthesized and incorporated into the middle of 25mer oligonucleotides using standard solid phase phosphoramidite chemistry. For

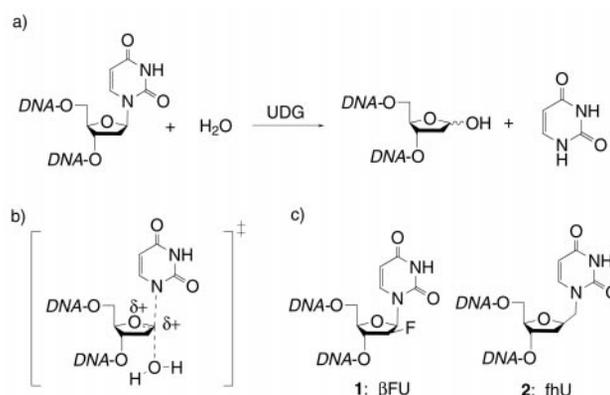


FIG. 1. The action of UDG and designed inhibitors of UDG. *a*, UDG catalyzes the hydrolysis of the *N*-glycosidic bond between uracil and the deoxyribose backbone. *b*, the proposed transition state for glycosidic bond cleavage by UDG, *c*, designed inhibitors of UDG.

double-stranded inhibitors, inhibitor oligonucleotides were annealed with complementary oligonucleotides containing a G opposite the analog.

Binding Interactions between HSV-1 and Human UDGs and the Inhibitors—The binding affinities of the HSV-1 and human UDGs for the designed inhibitors were evaluated by EMSA (Fig. 2). HSV-1 UDG binds the double-stranded (ds) β FU inhibitor tightly with an apparent K_d of 4 nM. Under the same experimental conditions, the ds β FU inhibitor fails to form a specific gel shift complex with human UDG. A 25mer containing inhibitor **2** was tested in the same way using EMSA and was found similarly to bind with high specificity to only the viral UDG, albeit with lower affinity ($K_d = 160$ nM).

UDGs are known to excise uracil from both single-stranded (ss) and dsDNA. We therefore tested the binding of HSV-1 and human UDGs to inhibitors **1** and **2** in ssDNA. Although HSV-1 UDG does not bind detectably to the ssfhUrd inhibitor, HSV-1 UDG retained nanomolar affinity for the ss β FdUrd inhibitor ($K_d = 47$ nM). Neither uracil analog in ssDNA bound the human UDG even at concentrations as high as 1 μ M. Thus, the β FdUrd inhibitor is exquisitely specific for the viral over the human UDG, irrespective of whether the DNA is single- or double-stranded.

Specific Inhibition of HSV-1 UDG Activity by β FdUrd and fhUrd Inhibitors—To determine whether binding of the analogs to UDG results in inhibition of catalytic activity, we analyzed the ability of the analogs to interfere with cleavage of a natural substrate by UDG. In this assay, a 5'- 32 P-labeled uracil-containing substrate is incubated with UDG in the presence or absence of unlabeled competitor analog. Processing of the natural substrate creates an abasic site, which undergoes strand cleavage to generate shortened oligonucleotide products upon treatment with aqueous piperidine. As shown in Fig. 3a, a 50-fold excess of ds β FU gives nearly complete inhibition (\sim 90%) of HSV-1 UDG activity (lane 4). The ss β FU oligonucleotide also inhibited HSV-1 UDG activity strongly (lane 7). Under the conditions of these assays, the fhUrd analog inhibited cleavage by \sim 40% in dsDNA (lane 5) but not to any significant extent in ssDNA (lane 8). By contrast, neither analog caused pronounced inhibition of human UDG, although β FU in both ssDNA and dsDNA showed evidence of modest inhibition ($<$ 25% relative to nonspecific DNA, Fig. 3b). Overall, the trends seen in cleavage inhibition assays closely parallel those in binding assays, consistent with the notion that these analogs target the enzyme active site.

Potential Uses for UDG Inhibitors—Here, we have described the design and evaluation of nanomolar inhibitors of HSV-1 UDGs. These UDG inhibitors have several potential applications. Although previous structural analyses of several UDGs,

² Y. Sekino, S. D. Bruner, and G. L. Verdine, unpublished results.

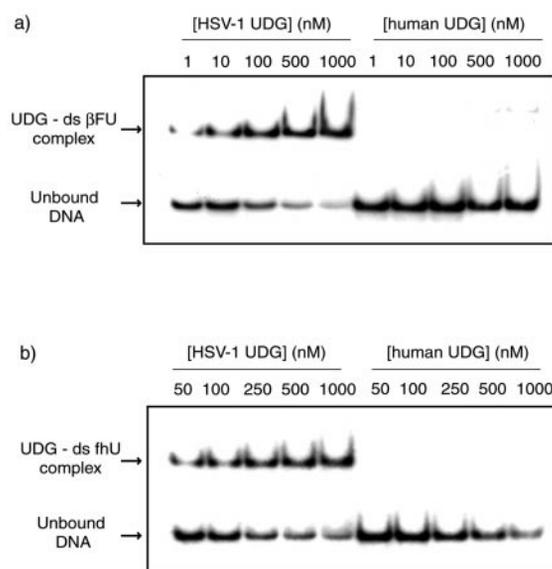


FIG. 2. EMSA assay comparing the binding affinities of HSV-1 and human UDg for dsβFUrd (a) and dsfHUrd (b). Concentration of 32 P-labeled oligonucleotides = 0.4 nM.

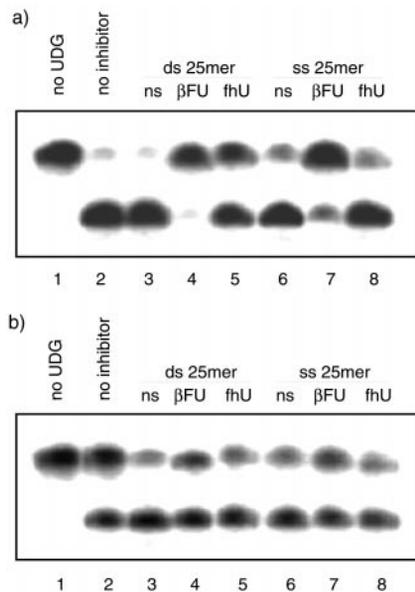


FIG. 3. Cleavage inhibition of HSV-1 UDg (a) and human UDg (b) using both single-stranded and double-stranded βFUrd and fHUrd analogs. Concentration of 32 P labeled uracil-containing substrate = 1 nM; concentration of competitor = 50 nM. ns, nonspecific competitor containing C in the place of U.

notably of human, *Escherichia coli*, and HSV-1 UDGs (4, 30–34), have elucidated certain key features of uracil excision repair, tight complexes of a native UDg enzyme with an intact DNA substrate or substrate analog have not previously been available for structural studies. The precise mechanism by which these enzymes scan the genome to locate and excise uracil residues remains unknown. The present inhibitors allow the formation of such stable complexes and are thus expected to shed further insight into the process of substrate recognition and catalysis by UDGs.

Viral infections represent a major human health concern with few therapeutic options available. The fact that many members of the poxvirus and herpesvirus families carry a functional UDg in their limited genome and that other viruses,

including the human immunodeficiency virus type 1, actively package host UDg protein into viral particles suggests a potentially important functional role for this enzyme (35). However, the precise function of UDGs in viral infection remains unclear. Highly selective and specific inhibitors described in this communication provide new reagents to probe the *in vivo* role of viral UDGs. Although oligonucleotides often suffer from poor cell permeability, there is reason to be optimistic in the case of these inhibitors, as oligonucleotides containing as few as 7 nucleotides inhibit HSV-1 UDg *in vitro*.²

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