Herpes simplex virus type 1 uracil-DNA glycosylase: isolation and selective inhibition by novel uracil derivatives

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We have purified *Herpes simplex* type 1 (HSV1) uracil-DNA glycosylase from the nuclei of HSV1-infected HeLa cells harvested 8 h post-infection, at which time the induction of the enzyme is a maximum. The enzyme has been shown to be distinct from the host enzyme, isolated from HeLa cells, by its lack of sensitivity to a monoclonal antibody to human uracil-DNA glycosylase. Furthermore, several uracil analogues were synthesized and screened for their capacity to discriminate between the viral and human uracil-DNA glycosylases. Both enzymes were inhibited by 6-(*p*-alkylanilino)uracils, but the viral enzyme was significantly more sensitive than the HeLa enzyme to most analogues. Substituents providing the best inhibitors of HSV1 uracil-DNA glycosylase were found to be in the order: *p*-

n-butyl < *p*-*n*-pentyl = *p*-*n*-hexyl < *p*-*n*-heptyl < *p*-*n*-octyl. The most potent HSV1 enzyme inhibitor, 6-(*p*-*n*-octylanilino)uracil (OctAU), with an IC_{50} of 8 μ M, was highly selective for the viral enzyme. Short-term [3 H]thymidine incorporation into the DNA of HeLa cells in culture was partially inhibited by OctAU, whereas it was unchanged when 6-(*p*-*n*-hexylanilino)uracil was present at concentrations that completely inhibited HSV1 uracil-DNA glycosylase activity. These compounds represent the first class of inhibitors that inhibit HSV1 uracil-DNA glycosylase at concentrations in the micromolar range. The results suggest their possible use to evaluate the functional role of HSV1 uracil-DNA glycosylase in viral infections and re-activation in nerve cells.

INTRODUCTION

Herpes simplex virus (HSV), after primary infection in peripheral mucocutaneous tissue, can penetrate nerve cells and establish latent infection in neuronal ganglia (Challberg and Kelly, 1989; Roizman and Sears, 1990). Re-infection with the same virus type has been described in humans, but it is extremely rare; most recurrent infections represent re-activation of the same virus type from latency. Although the molecular mechanism leading to viral re-activation is still unclear, it is known that re-activation is the viral answer to environmental injury to the host cell. For example, ultraviolet light, trauma to neuronal ganglia, immunosuppression or general stress situations could damage the host cell and seriously compromise virus survival (Roizman and Sears, 1990). Starting in the lytic cycle, HSV suppresses host DNA and protein synthesis (Roizman and Sears, 1990) and induces its enzymic machinery controlling the efficient biosynthesis of DNA precursors, the replication of its genome, and the maturation of the virus particle.

Among the variety of enzymes induced by HSV during the re-activation event, some replace the suppressed host counterparts, for instance dUTPase (Caradonna and Cheng, 1981), and others supply cellular enzymes that are lacking. The enzymes of this second class are either those catalysing specific viral reactions, such as virus assembly, or those whose cellular counterparts are developmentally absent, such as DNA polymerases and thymidine kinase (TK).

Our laboratory has demonstrated that adult neurons lack not only TK, but also replicative DNA polymerases α (Hübscher et al., 1977, 1978) and δ/ϵ (Spadari et al., 1988), and uracil-DNA

glycosylase (Focher et al., 1990). In neurons, the activity of these enzymes decreases during fetal development and disappears at birth, in synchrony with the pattern of neural division (Hübscher et al., 1977, 1978; Spadari et al., 1988; Focher et al., 1990). This means that in neurons, where viral re-activation takes place, HSV replication strictly depends on its own enzymes. This is supported by the observation that TK[−] HSV can sustain lytic infections in mucosal and skin surfaces (TK⁺ cells), but fails to re-activate from adult explanted ganglia (TK[−] cells).

The pivotal role of HSV TK in re-activation was clearly demonstrated by the use of non-substrate inhibitors of viral TK synthesized and characterized in our laboratories (Focher et al., 1988; Spadari and Wright, 1989). Used in a murine model, these compounds significantly reduced the number of latently infected trigeminal ganglia that yielded virus upon explant culture (Leib et al., 1990).

Induction of HSV uracil-DNA glycosylase activity was observed during viral infection (Caradonna and Cheng, 1981), and the coding sequence of the viral enzyme has been identified (Caradonna et al., 1987; Mullaney et al., 1989; Worrall and Caradonna, 1988). Uracil-DNA glycosylase belongs to the class of enzymes involved in post-replicative DNA repair processes. Its function consists of the specific removal of uracil residues from DNA, deriving either from cytosine deamination or dUTP incorporation, by cleavage of the N-glycosidic bond linking the base to the deoxyribose phosphate backbone.

In this work, we present details of the isolation and characterization of the enzyme from HSV1-infected cells, and report the first specific inhibitors of HSV1 uracil-DNA glycosylase acting in the micromolar range. These compounds

Abbreviations used: HSV1, *Herpes simplex virus* type 1; TK, thymidine kinase; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; NP-40, Nonidet P-40; DMEM, Dulbecco's modified Eagle's medium; BuAU, 6-(*p*-*n*-butylanilino)uracil; OctAU, 6-(*p*-*n*-octylanilino)uracil; HexAU, 6-(*p*-*n*-hexylanilino)uracil.

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might be valuable in defining whether this enzyme, like the viral TK, plays a key role in viral re-activation from normal UDG⁻ nerve cells.

MATERIALS AND METHODS

Chemicals and enzymes

Deoxyribonucleoside triphosphates were from Boehringer or, for ³H-labelled nucleotides and nucleosides, from Amersham. Heparin-Sepharose CL-6B was from Pharmacia, pepstatin A and phenylmethanesulphonyl fluoride (PMSF) were from Sigma, BSA (A grade) was from Calbiochem. Sodium metabisulphite and plastic t.l.c. sheets (polyethyleneimine-cellulose F; 0.1 mm) were from Merck. Nonidet P-40 was from BDH, and dithiothreitol (DTT) was from Fluka. Phosphocellulose P11 and DE-32 were from Whatman. DNA polymerase I, Klenow fragment, was purchased from Boehringer.

HSV1 infection and harvesting of infected cells

Confluent monolayers of HeLa BU (TK⁻) cells on 225 cm² flasks were infected with HSV1 strain F (Ejercito et al., 1968) at a multiplicity of infection of 5 plaque-forming units/cell. After a 1 h adsorption period, the monolayers were rinsed twice with phosphate-buffered saline, overlaid with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, and incubated for 8 h. The cells were then detached with a rubber policeman, the suspension was divided into portions in plastic tubes, and cells were kept frozen at -70 °C until use.

Uracil-DNA glycosylase assay

Assays in a final volume of 25 µl each contained 100 mM Tris/HCl (pH 8.0), 5 mM DTT, 10 mM EDTA, 500 ng of [³H]dUMP-labelled DNA (40 c.p.m./ng; 220 c.p.m./pmol of uracil; 3.6 µM uracil) prepared as described (Focher et al., 1990), 4 µg of unlabelled activated DNA and the enzyme (0.3 unit) to be tested (when HSV1 enzyme was used, BSA was added in the test tube in order to obtain a protein concentration comparable with that for the human enzyme). After incubation at 37 °C for 30 min, 20 µl portions of the mixtures were spotted on to GF/C filters (Whatman). The filters were washed three times in 5% (v/v) trichloroacetic acid for 5–10 min and twice in ethanol. The filters were dried and the acid-insoluble radioactivity was measured by scintillation counting in 1 ml of scintillation fluid. One unit of uracil-DNA glycosylase removes 1 nmol of uracil from DNA in 1 h at 37 °C.

Purification of HSV1 uracil-DNA glycosylase

HeLa cells (5 × 10⁸) were infected by HSV1 as described above and collected 8 h post-infection. The cells were resuspended in 6 vol. of ice-cold buffer A (10 mM NaCl, 1 mM potassium phosphate, pH 6.8, 1 mM DTT and 1% dimethyl sulphoxide, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). After 5 min on ice the cells were homogenized in a Dounce homogenizer. The homogenate was centrifuged at 1400 g for 15 min in order to precipitate the nuclei. The nuclei were suspended in 5 vol. of buffer B (1 mM potassium phosphate, pH 7.0, 0.32 M sucrose, 1 mM MgCl₂, 0.3% NP-40 detergent and 1 mM DTT, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). In this buffer the nuclei were homogenized and centrifuged at 1400 g for 15 min. The nuclear pellet was resuspended in 5 vol. of buffer C (400 mM potassium phosphate, pH 7.0, 1 mM DTT, 0.1% NP-40 and 1% dimethyl sulphoxide, containing 1 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). After 5 min on ice the nuclei

were disrupted in a Dounce homogenizer. The homogenate was centrifuged at 48000 g for 1 h. The supernatant was loaded on a DE-32 column (4 ml) previously equilibrated with 10 column volumes of buffer C (to remove nucleic acids). The flow-through and the first 20 ml of column wash with buffer C were collected and dialysed against three changes of buffer D (20 mM potassium phosphate, pH 7.5, 1 mM DTT and 0.1% NP-40, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin) in order to lower the ionic strength. The dialysed solution was loaded on to a DE-32 column (4 ml) previously equilibrated with buffer D. The flow-through and the first 16 ml of the column wash with buffer D containing the enzyme activity were loaded on to a phosphocellulose column (2 ml) equilibrated with buffer E (20 mM potassium phosphate, pH 7.5, 20% glycerol, 1 mM DTT and 0.1% NP-40, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). The phosphocellulose column was washed with 4 column volumes of buffer E, and the enzyme was eluted with 20 ml of a linear gradient from 20 to 400 mM potassium phosphate (pH 7.5) in buffer E. The pooled fractions, which eluted at approx. 200 mM potassium phosphate, were dialysed against buffer F (20 mM potassium phosphate, pH 7.0, 30% glycerol, 1 mM DTT, 0.5 mM EDTA and 0.1 mM EGTA, containing 0.2 mM PMSF and 1 µM pepstatin), and then loaded on to a heparin-Sepharose column (1 ml) equilibrated with buffer F. The column was washed with 4 ml of buffer F, and the enzyme was eluted with 18 ml of a linear gradient from 0 to 600 mM KCl in buffer F; the enzyme eluted at 250 mM KCl. The pooled fractions were immediately frozen and stored in liquid nitrogen in small aliquots. The final preparation had a specific activity of 11080 units/mg and there was no contamination by nucleases. The sedimentation coefficient was 3.17. The purification procedure is summarized in Table 1.

Purification of human uracil-DNA glycosylase

Human uracil-DNA glycosylase was purified from the cytoplasm of HeLa cells by a procedure partially derived from that of Krokan and Wittwer (1981). HeLa-S3 cells (2 × 10⁹) were resuspended in 28 ml of ice-cold buffer G (10 mM Tris/HCl, pH 7.5, 3 mM MgCl₂, 2 mM EGTA and 1 mM DTT, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). After 5 min on ice the cells were homogenized in a Dounce homogenizer, and then 14 ml of buffer H (340 mM Tris/HCl, pH 8.1, 3 mM MgCl₂, 150 mM glucose, 2 mM EGTA, 0.15% Triton X-100 and 1 mM DTT, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin) was added. The resulting solution was centrifuged at 680 g for 5 min. The supernatant was saved, and the pellet containing the nuclei was washed in a Dounce homogenizer in 8 ml of buffer I (buffer G/buffer H, 2:1, v/v) and then centrifuged at 680 g for 5 min. The supernatant was combined with the previous one and centrifuged at 100000 g for 1 h. The derived supernatant was dialysed for 8 h against 20 vol. of buffer J (20 mM Tris/HCl, pH 7.5, 20% glycerol, 0.1% Triton X-100 and 1 mM DTT, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin) and then loaded on to a DE-32 column (10 ml) equilibrated with buffer J. The flow-through and the column wash with buffer J containing the enzyme activity were loaded on to a phosphocellulose column (5 ml) equilibrated with buffer K (20 mM potassium phosphate, pH 7.5, 20% glycerol, 0.1% Triton X-100 and 1 mM DTT, containing 0.2 mM PMSF, 4 mM sodium metabisulphite and 1 µM pepstatin). The column was washed with 10 column volumes of buffer K, and the enzyme was eluted with a linear gradient (50 ml) of 20–300 mM potassium

Table 1 Purification of HSV1 uracil-DNA glycosylase

	Protein (mg)	Activity (units)	Specific activity (u/mg)	Recovery (%)	Purification (-fold)
Nuclear extract	20	1640	82	—	—
48 000 <i>g</i> centrifugation	4.62	1530	331	93	4.0
DEAE (I)	4.24	2328	549	142	6.7
DEAE (II)	1.08	2268	2100	138	25.6
Phosphocellulose P11	0.28	950	3386	58	41.3
Heparin-Sepharose	0.05	554	11 080	34	135.1

Table 2 Yields and properties of 6-(*p*-alkylanilino)uracils

Compounds were synthesized by reaction between 6-chlorouracil and the aniline in refluxing 2-methoxyethanol as described (Wright and Brown, 1980). Abbreviations: EtOH, ethanol; HOAc, glacial acetic acid. C, H and N analyses agreed to within $\pm 0.4\%$ of calculated values. The general formula of the derivatives is given below:

R	Yield (%)	Melting point (°C)	Crystallization solvent	Formula
Isobutyl	78	327–333	EtOH	C ₁₄ H ₁₇ N ₃ O ₂
Isopentyl	88	319–320	EtOH	C ₁₅ H ₁₉ N ₃ O ₂
n-Pentyl	67	321–324	EtOH	C ₁₅ H ₁₉ N ₃ O ₂
n-Hexyl	70	317–321	EtOH	C ₁₆ H ₂₁ N ₃ O ₂ · $\frac{1}{2}$ EtOH
n-Heptyl	47	303–304	EtOH	C ₁₇ H ₂₃ N ₃ O ₂ · $\frac{3}{8}$ EtOH
n-Octyl	52	298–301	HOAc	C ₁₈ H ₂₅ N ₃ O ₂

phosphate, pH 7.5, in buffer K. The pooled fractions (15 ml) were dialysed against 500 ml of buffer L (20 mM potassium phosphate, pH 7.0, 30% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.2 mM PMSF and 1 μ M pepstatin) and then loaded on to a heparin-Sepharose column (1 ml) equilibrated with buffer L. The column was washed with 20 ml of buffer L, and the enzyme was eluted with a linear gradient (10 ml) of 0–600 mM KCl in buffer L. The enzyme was further purified as follows: 100 μ l portions of the pooled fractions from the heparin-Sepharose column were desalted on a Sephadex G-50 (fine) column and then loaded on a poly(U)-Sepharose column (50 μ l) equilibrated in 50 mM Tris/HCl, pH 7.5, 2 mM DTT, 10% glycerol and 0.5 mM PMSF. The column was washed with the same buffer and then with increasing concentrations of KCl in the same buffer. Enzyme, eluted at 400 mM KCl, was immediately frozen and stored in small aliquots in liquid nitrogen. The final preparation had a specific activity of 1060 units/mg and there was no contamination by nucleases. It had a sedimentation coefficient of 3.5.

Slot-blot analysis

The same number of units (2.5 units) or the same amount of proteins (250 ng) of HSV1 and human uracil-DNA glycosylases were loaded on a nitrocellulose filter in a slot-blot filtration

manifold (Hoefer). After a wash with phosphate-buffered saline, the filter was removed and processed as described (Towbin et al., 1979). The blot was tested with 1 μ g/ml anti-(human uracil-DNA glycosylase) monoclonal antibody (42.08.07). Anti-(mouse IgG) conjugated with biotin was added, and the blot was developed with streptavidin-alkaline phosphatase reagent (Bio-Rad).

[³H]Thymidine incorporation into DNA of HeLa cells

Cells were grown in suspension at 37 °C in DMEM containing 10% fetal calf serum. At a density of 0.6×10^6 /ml cells were collected by centrifugation and suspended in fresh medium without fetal calf serum at a density of 10^6 /ml. Aliquots of 0.4×10^6 cells were incubated at 37 °C in the presence of 40 μ M [³H]thymidine (25 μ Ci/ml; 25 Ci/mmol) in the absence or presence of inhibitors at various concentrations. At 0, 15, 30 and 45 min 80 μ l samples of culture were spotted on to 25 mm GF/C filters (Whatman). The filters were washed immediately in a large volume of ice-cold 5% trichloroacetic acid. The filters were washed twice in trichloroacetic acid and twice in ethanol, dried and counted in Omnifluor scintillation fluid.

Synthesis of inhibitors

The syntheses of several of the compounds tested have been described (Baker and Rzeszutarski, 1968; Wright and Brown, 1980). New 6-(*p*-alkylanilino)uracils were prepared by reaction between 6-chlorouracil and the *p*-alkylanilines (Aldrich Chemical Co.) in refluxing 2-methoxyethanol as described (Wright and Brown, 1980). Yields and properties of new compounds are presented in Table 2. Proton n.m.r. spectra of all compounds in [²H₆]dimethyl sulphoxide (300 MHz; Varian Unity 300 instrument) were fully consistent with the proposed structures.

RESULTS

Induction of HSV1 uracil-DNA glycosylase

HSV1-infected HeLa cells were collected at 0, 2, 4, 6, 8, 10, 12 and 18 h post-infection, and their cytoplasmic and nuclear fractions were tested for uracil-DNA glycosylase activity. Most of the enzyme activity was present in the nuclear fraction (results not shown) with a peak at 8 h after infection (Figure 1), indicating a viral induction of the enzyme.

Purification of HSV1 uracil-DNA glycosylase

The enzyme was purified from the nuclei of HSV1-infected HeLa cells at the peak of enzyme induction, as described in the Materials and methods section, and Table 1 summarizes the yields and degree of purification obtained. Since most of the inducible uracil-DNA glycosylase activity was observed in the nuclei we used only the nuclear fraction as starting material. The purification sequence resulted in an enzyme preparation with a specific activity of 11 080 units/mg and there was no contamination by nucleases. For comparative studies, the human uracil-DNA glycosylase was purified from HeLa cells based on the method of Krokan and Wittwer (1981) (see the Materials and methods section). The latter preparation had a specific activity of 1060 units/mg, with no contamination by nucleases. The lower specific activity of the human compared with the viral enzymic preparation may be due to: (i) the 5-fold higher protein concentration of the human crude extract derived from the cellular cytoplasm, (ii) the low levels of human uracil-DNA glycosylase activity in non-infected cells compared with the high levels of the viral-induced activity at the time of induction, and (iii) the

Figure 1 Induction of uracil-DNA glycosylase activity in nuclear extracts of HeLa cells after HSV1 infection

At each time point, 2×10^7 cells were collected, sonicated three times at 100 W in 3 vol. of 10 mM Tris/HCl, pH 7.5, 10 mM KCl, 1 mM DTT and 0.1 mM EDTA, containing 1 mM PMSF and 1 μ M pepstatin, and centrifuged at 8000 *g* for 10 min. The supernatant is the cytoplasmic fraction. The pellet, containing most of the nuclei, was made 400 mM in KCl in 3 vol. of the same buffer. Sonication and centrifugation were repeated as above. The resulting supernatant is the nuclear fraction.

Figure 2 SDS/PAGE of human (0.5 μ g; lane H) and HSV1 (0.25 μ g; lane HSV) uracil-DNA glycosylases

Electrophoresis was conducted in an SDS/10%-polyacrylamide gel, and proteins were stained with Coomassie Blue. Lane M, protein standards: 1 μ g each of phosphorylase *b* (92.5 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa).

greater stability of the viral uracil-DNA glycosylase during the purification. The electrophoretic pattern of the viral enzyme was compared with that of the human enzyme by SDS/PAGE (Figure 2). Both enzymes showed a band at a molecular mass of 37 kDa, but the human enzyme had higher molecular mass bands as well. This apparent molecular mass of 37 kDa is consistent with the reported molecular masses of 39 kDa for the HSV1 enzyme (Caradonna et al., 1987) and 37 kDa for the human placental enzyme (Seal et al., 1987).

That the uracil-DNA glycosylase isolated from virus-infected HeLa cells is indeed viral in origin was demonstrated by a slot-blot experiment with a monoclonal antibody to the human placenta enzyme (Arenaz and Sirover, 1983). Figure 3 shows that

Figure 3 Slot-blot analysis of HSV1 (lane HSV) and human (lane H) uracil-DNA glycosylases

A, 2.5 units of each enzyme; B, 0.22 μ g of total protein of each enzyme preparation; C, 2.5 units of each enzyme in 2.3 μ g of total protein (HSV1 enzyme preparation was complemented with BSA). The filter was incubated in the presence of 10 μ g of monoclonal anti-(human uracil-DNA glycosylase) antibody 42.08.07 (Arenaz and Sirover, 1983), kindly supplied by Professor M. Sirover, as described in the Materials and methods section.

Table 3 Effects of 6-anillinouracils on HSV1 and human uracil-DNA glycosylases

The IC₅₀ is the concentration of compound that caused half-maximal inhibition of [³H]uracil release from [³H]dUMP-containing DNA. Assays were performed as described in the Materials and methods section in the presence of various concentrations of test compounds. Control assays contained the same concentration of the compound solvent, dimethyl sulphoxide. The general formula is given below:

Substituent(s)	IC ₅₀ (μ M)	
	HSV1	Human
4-Hydroxy	> 500	> 500
3,4-Dimethoxy	> 500	> 500
3,4-Dichloro	500	> 500
3-Ethyl-4-methyl	500	> 500
4-n-Propyl	> 500	> 500
4-Isopropyl	> 500	> 500
4-n-Butyl (BuAU)	150	> 500
4-Isobutyl	400	> 500
4-n-Pentyl	30	250
4-Isopentyl	140	200
4-n-Hexyl (HexAU)	30	> 300
4-n-Heptyl	20	140
4-n-Octyl (OctAU)	8	> 300

the human HeLa enzyme, but not the HSV1 enzyme, reacted with the monoclonal antibody 42.08.07.

Uracil analogues discriminate between human and HSV1 uracil-DNA glycosylases

Screening of a large number of uracil derivatives and related compounds against the purified HSV1 and human HeLa uracil-

Figure 4 Dose-response curves for uracil-DNA glycosylase inhibition by 6-(*p*-*n*-alkylanilino)uracils

The HSV1 (○) and human (●) enzymes were assayed as described in the Materials and methods section with the addition of stock solutions of inhibitors dissolved in dimethyl-sulphoxide. Control activity corresponds to enzyme activity in the presence of an identical concentration of the solvent. PentAU, 6-(*p*-*n*-pentylanilino)uracil; HeptAU, 6-(*p*-*n*-heptylanilino)uracil.

DNA glycosylases revealed several 6-anilinouracils that showed weak activity against the viral enzyme. Many substituted derivatives were only marginally active at 500 μM in inhibiting the release of [^3H]uracil in the standard uracil-DNA glycosylase assay (see the Materials and methods section), but 6-(*p*-*n*-butylanilino)uracil (BuAU) inhibited the viral enzyme by 50% at 150 μM and was ineffective at 500 μM against the human enzyme. This result prompted the synthesis and testing of additional *p*-alkyl derivatives in an attempt to identify more potent and selective inhibitors of the HSV1 enzyme. The properties of these new compounds are summarized in Table 2. The results of testing of representative 6-anilinouracils and the new derivatives are summarized numerically in Table 3, and the selectivity of the more potent inhibitors is displayed graphically in Figure 4. 6-Anilinouracils with *n*-alkyl groups in the *p* position of the anilino ring were progressively more potent as inhibitors of the HSV1 enzyme and retained a high degree of selectivity for the viral enzyme. The most potent compound, 6-(*p*-*n*-octylanilino)uracil (OctAU) had an IC_{50} of 8 μM against the viral enzyme, but one of $> 300 \mu\text{M}$, the highest concentration tested, against the human enzyme. Interestingly, the *n*-heptyl derivative was less selective than the *n*-hexyl or *n*-octyl derivatives (Figure 4). Both the *n*-hexyl and *n*-octyl derivatives were found to be inactive against viral TK and DNA polymerase (results not shown).

Mechanism of inhibition

Insight into the mechanism by which 6-(*p*-alkylanilino)uracils inhibit HSV1 uracil-DNA glycosylase was sought by studying the dependence of inhibition on the concentration of the substrate in the reaction. The effect of OctAU at varying concentrations of uracil, as dUMP concentration in DNA, are displayed in the double-reciprocal plot in Figure 5. The results suggest that the

Figure 5 Double-reciprocal plot of the inhibition of HSV1 uracil-DNA glycosylase by OctAU as a function of substrate concentration

Assays were done as described in the legend to Figure 4 in the presence of various concentrations of [^3H]dUMP-labelled DNA. Concentrations of OctAU used: ○, 0 μM ; ●, 10 μM ; ▽, 16 μM .

inhibition is competitive with the substrate, perhaps as a result of inhibitor binding to the catalytic site of the enzyme. This contrasts with the reported non-competitive effect of uracil itself as an inhibitor of human uracil-DNA glycosylase (Caradonna and Cheng, 1981). Uracil probably represents a product inhibitor of

Figure 6 Effect of 6-(*p*-*n*-alkylanilino)uracils on short-term incorporation of [3 H]thymidine into DNA of HeLa cells in culture

Cells were incubated at 37 °C in medium containing inhibitors or inhibitor solvent and [3 H]thymidine. Aliquots were removed at various times and processed as described in the Materials and methods section. Maximum incorporation at 45 min corresponded to 8.37 pmol/ 10^6 cells.

uracil-DNA glycosylase, whereas the anilinouracils may bind to the enzyme as analogues of the substrate.

Effect of uracil analogues on short-term [3 H]thymidine incorporation into DNA of HeLa cells

Two of the most potent inhibitors of HSV1 uracil-DNA glycosylase, 6-(*p*-*n*-hexylanilino)uracil (HexAU) and OctAU, were tested with cultured HeLa cells in order to evaluate their effect on DNA synthesis. Experiments were performed as described in the Materials and methods section to evaluate short-term [3 H]thymidine incorporation into the DNA of untreated and inhibitor-treated HeLa cells. The results depicted in Figure 6 show that HexAU did not affect DNA synthesis by HeLa cells at 300 μ M, but OctAU inhibited DNA synthesis even at 5 μ M. The apparent IC_{50} for the effect of OctAU was about 25 μ M, but even concentrations as high as 300 μ M did not completely suppress thymidine incorporation (Figure 6). The effect of OctAU on cellular DNA synthesis is similar to that previously reported for the analogue BuAU, a selective inhibitor of DNA polymerase α (Wright et al., 1980). BuAU inhibited the incorporation of [3 H]thymidine by HeLa cells in culture, but not incorporation of labelled uridine or leucine, markers for RNA and protein synthesis respectively. Thus, although OctAU is a more potent inhibitor of HSV1 uracil-DNA glycosylase than HexAU, the latter compound lacks an effect on cellular DNA synthesis at concentrations at which the viral enzyme is strongly inhibited.

The unexpected observation that OctAU inhibited [3 H]-thymidine incorporation by HeLa cells in culture, and the similarity of its structure to that of a selective inhibitor of DNA polymerase α , BuAU (Wright et al., 1980), prompted us to ask if this new derivative inhibited one or more of the eukaryotic replicative DNA polymerases α , δ and ϵ . In standard DNA polymerase reactions with nicked DNA, assayed as described (Weiser et al., 1991), OctAU had little effect on HeLa DNA polymerase α ($IC_{50} > 500 \mu$ M), but inhibited HeLa DNA polymerase ϵ with an IC_{50} of 80 μ M. HexAU, on the other hand, weakly inhibited both enzymes ($IC_{50} > 500 \mu$ M) under these conditions, thus explaining the lack of effect of this compound on cellular DNA synthesis. Furthermore, both DNA polymerases ϵ and δ [proliferating cell nuclear antigen (PCNA)-dependent]

from calf thymus were more sensitive to OctAU than polymerase α (results not shown). These results suggest that OctAU may represent a prototype of selective inhibitors of DNA polymerase δ and/or ϵ , just as BuAU was the prototype of a series of useful selective inhibitors of DNA polymerase α (Brown et al., 1986).

DISCUSSION

The DNA repair enzyme uracil-DNA glycosylase has been purified from HSV1-infected HeLa cells. By comparison with the enzyme isolated from uninfected HeLa cells, this enzyme has been shown to be of viral origin by virtue of: (1) its time-dependent induction upon virus infection of cells (Figure 1); (2) its apparent molecular mass of 37 kDa (Figure 2), and (3) its insensitivity to a monoclonal antibody to the human enzyme (Figure 3). The enzyme further differs from its cellular counterpart in its potent and selective inhibition by 6-(*p*-*n*-alkylanilino)uracils (Table 3), compounds that appear to act mechanistically as substrate analogues. The enzyme isolated from HeLa cells was consistently less sensitive to inhibition by all compounds in this series.

Few inhibitors of uracil-DNA glycosylases have been reported. Among a group of simple pyrimidines, only uracil was found to inhibit the enzyme from leukaemic blast cells (Caradonna and Cheng, 1980); uracil caused 81% inhibition at 1 mM, and was non-competitive with the substrate (as [dUMP]). Uracil, 6-aminouracil and 5-azauracil were reported to inhibit uracil-DNA glycosylase from HeLa cells, but with IC_{50} values of 1–2 mM (Krokan and Wittwer, 1981). In contrast, 5-fluorouracil and 5-bromouracil at 1 mM inhibited uracil-DNA glycosylase from human placenta only after preincubation with the enzyme at 4 °C (Seal et al., 1987). Neither the 2'-deoxyribonucleosides nor the corresponding 5'-monophosphates of the 5-halouracils substantially inhibited the placenta enzyme even after the preincubation period. The results in the present paper reveal the first potent and selective inhibitors of the viral uracil-DNA glycosylase that can be used as probes of the mechanism of enzyme action, and these may prove to be useful in studying the role of this enzyme in virus growth and re-activation.

It is known that uracil can arise in DNA by two mechanisms: (i) the misincorporation of dUMP residues by DNA polymerases,

and (ii) the spontaneous deamination of cytosine. In particular with regard to the nervous system, where herpes viruses establish latency, it is worthwhile to recall that the only DNA polymerase present in adult neurons, DNA polymerase β , in contrast to the replicative DNA polymerases α and ϵ (which utilize dUTP 70–80% less efficiently than dTTP), does not discriminate between dUTP and dTTP (Focher et al., 1990). This suggests that dUTP could be introduced into DNA during DNA repair in adult neurons, although most of the uracil in DNA results from deamination of cytosine. These observations suggest that dUMP residues may accumulate in DNA during the life-span of adult neurons (Mazzarello et al., 1990).

The continuous spontaneous deamination of cytosine, the fact that both viral DNA polymerase (Focher et al., 1992) and DNA polymerase β (Focher et al., 1990) incorporate dUTP and dTTP with comparable efficiency, and the lack of cellular uracil-DNA glycosylase in nerve cells (Focher et al., 1990) suggest a role of the virus-encoded uracil-DNA glycosylase in the re-activation and replication of *Herpes simplex* virus in nerve cells. This role is supported by our finding that uracil in DNA affects specific DNA-protein interactions (Verri et al., 1990; Focher et al., 1992).

In conclusion, we hypothesize that HSV1 uracil-DNA glycosylase, which is non-essential for the proliferation of HSV1 in cell cultures, could play a key role in nerve cells in 'cleansing' of the viral genome before DNA replication as well as in the removal of misincorporated uracil during viral DNA replication. We intend to use the specific inhibitors described in this paper in order to verify the role of viral uracil-DNA glycosylase during virus re-activation and replication in nerve cells *in vivo*. One derivative, HexAU, by virtue of its lack of effect on cellular DNA synthesis and on viral TK and DNA polymerase at concentrations that completely inhibit the viral enzyme, has been selected for such studies.

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