0026-895X/90/050658-07$02.00/0

MOLECULAR PHARMACOLOGY, 37:658–664

Mechanism of Action of 5-(2-Chloroethyl)-2′-Deoxyuridine, a Selective Inhibitor of Herpes Simplex Virus Replication*

ERIK DE CLERCQ, RIA BERNAERTS, ALES MERTA, and BRIGITTE ROSENWIRTH

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium (E.D.C., R.B.), the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 16610 Prague 6, Czechoslovakia (A.M.), and Sandoz Forschungsinstitut, A-1235 Vienna, Austria (B.R.)

Received November 17, 1989; Accepted February 2, 1990

SUMMARY

5-(2-Chloroethyl)-2′-deoxyuridine (CEDU) is a potent and selective inhibitor of the replication of herpes simplex virus type 1 (HSV-1). CEDU is preferentially phosphorylated by HSV-infected (Vero) cells, as compared with mock-infected cells or cells infected with a thymidine kinase-deficient strain of HSV-1. The end product of this phosphorylation process, CEDU 5′-triphosphate, is a competitive inhibitor of HSV-1 DNA polymerase activity and, to a lesser extent, of cellular DNA polymerase α activity. However, in the absence of the natural substrate dTTP, CEDU 5′-triphosphate also serves as an alternative substrate for viral and cellular DNA polymerase. When exposed to HSV-1-infected cells, [2-^{14}C]CEDU was incorporated into both viral and cellular DNA. The extent to which [2-^{14}C]CEDU was incorporated remained approximately constant over a concentration range of 0.5 to 50 μM. Within this concentration range, CEDU effected a concentration-dependent inhibition of viral DNA synthesis that closely paralleled the inhibition of viral progeny formation. It is postulated that CEDU owes (i) its selectivity as an antiviral agent to its preferential phosphorylation by the virus-infected cell and (ii) its antiviral potency to an inhibition of viral DNA synthesis at the level of the viral DNA polymerization reaction.

Various 5-substituted pyrimidine nucleoside analogues have been identified as antiherpes agents (1). Two of these compounds, i.e., 5-iodo-2′-deoxyuridine (idoxuridine) and 5-trifluoromethyl-2′-deoxyuridine (trifluridine), have been used worldwide for the topical treatment of herpetic eye infections and a third one, i.e. EDU is on the market in the Federal Republic of Germany, also for the topical treatment of herpetic keratitis. The great potential of this class of compounds is attested to by one of its most potent and selective congeners, BVDU, which *in vitro* inhibits the replication of HSV-1 and varicella zoster virus at a concentration as low as 0.01 μM, while not being toxic to the host cells up to a concentration of 100 μM or higher (2).

Griengl *et al.* (3) recently described a series of 5-haloalkyl pyrimidine nucleoside analogues, from which CEDU emerged as the most potent antiviral agent; although less potent than BVDU *in vitro*, CEDU showed efficacy against HSV-1 infections *in vivo* at doses that were equal to (upon topical administration) or lower than [upon systemic (oral or intraperitoneal) administration] those at which BVDU was active (4–6). In the different experimental HSV-1 infections in which the efficacy of CEDU was compared with that of acyclovir, CEDU invariably proved to be superior to acyclovir (4).

Little is known about the mode of action of CEDU. The compound is inactive against TK$^-$ mutants of HSV-1 (7),[1] which means that the compound must be phosphorylated by the virus-encoded TK to be antivirally active. On the other hand, CEDU also acts as a substrate for pyrimidine nucleoside phosphorylases, e.g., thymidine phosphorylase, which cleave the *N*-glycosidic linkage and, thereby, release the free pyrimidine base CEU (8). The latter is, as such, inactive against HSV. Furthermore, CEDU may be converted, even upon prolonged storage at 4°, to its furanyl derivative 5-(2-deoxy-β-D-ribosyl)-2,3-dihydro-5*H*-furano[2,3-*d*]pyrimidin-6-one [originally referred to as compound **X** (6) and later characterized as compound **5** (3)]. This furanyl derivative has no antiviral activity (3).

To obtain a better insight into the mechanism of action of CEDU and its metabolism by the cells, radiolabeled [2-^{14}C]

This work was supported by Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek Grant 3.0040.83 and Belgian Geconcerteerde Onderzoeksacties Grant 85/90-79.

[1] B. Rosenwirth, unpublished data.

ABBREVIATIONS: EDU, 5-ethyl-2′-deoxyuridine; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; CEDU, 5-(2-chloroethyl)-2′-deoxyuridine; CEDUMP, 5-(2-chloroethyl)-2′-deoxyuridine 5′-monophosphate; CEDUTP, 5-(2-chloroethyl)-2′-deoxyuridine 5′-triphosphate; CEU, 5-(2-chloroethyl)uracil; EDUTP, 5-ethyl-2′-deoxyuridine 5′-triphosphate; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; HSV-1, herpes simplex virus type 1; TK$^-$, thymidine kinase-deficient; PFU, plaque-forming unit; TK, thymidine kinase.

CEDU was synthesized. Its phosphorylation pattern was followed in both virus-infected and uninfected cells and, after [2-^{14}C]CEDU was found to be specifically phosphorylated by virus-infected cells, these cells were analyzed to assess the incorporation of [2-^{14}C]CEDU into both viral and cellular DNA. CEDU achieved a dose-dependent reduction in virus yield, which closely paralleled, and most likely resulted from, an inhibitory effect of the compound on viral DNA synthesis. The latter could be attributed, at least in part, to a competitive inhibition of the viral DNA polymerase by CEDUTP.

Experimental Procedures

Compounds. CEDU was synthesized at the Sandoz Forschungsinstitut by the method described by Griengl *et al.* (3). [2-^{14}C]CEDU was synthesized by H. Andres (Sandoz AG, Basel, Switzerland). The seven-step synthesis started from barium [^{14}C]carbonate. The label was introduced into position 2 of the pyrimidine moiety by condensation of [^{14}C]urea with the sodium salt of hydroxymethylene-γ-butyrolactone, leading to 5-(2-hydroxyethyl)-(1*H*, 3*H*)-[2-^{14}C]pyrimidine-2,4-dione, which was subsequently coupled with a protected furanosyl group. Transformation of the hydroxyethyl group into the chloroethyl group and deprotection with sodium ethoxide finally gave pure 5-(2-chloroethyl)-1-(2-desoxy-β-D-erythropentofuranosyl)-(1*H*,3*H*)-[2-^{14}C]pyrimidine-2,4-dione ([2-^{14}C]CEDU). The specific radioactivity of [2-^{14}C]CEDU was 18 mCi/mmol. EDU was kindly provided by E. Mauz and B. Hempel (Robugen GmbH Pharmazeutische Fabrik, Esslingen/Neckar, FRG). EDUMP and CEDUMP were prepared from EDU and CEDU following the method of Yoshikawa *et al.* (9); EDUTP and CEDUTP were prepared from the corresponding 5′-monophosphates by the method of Hoard and Ott (10). The syntheses of these 5′-triphosphates were accomplished at the Rega Institute by L. Kerremans and P. Herdewijn. The 5′-triphosphate of [*methyl*-^{3}H]thymidine (specific activity, 30 Ci/mmol) was obtained from Amersham International, as were [8-^{3}H]dATP (specific activity, 25 Ci/mmol) and [^{32}P]orthophosphate (specific activity, 3000 Ci/mmol).

Cells. Vero (African green monkey kidney) cells were grown to confluence (1–2 × 10^{6} cells) in polystyrene 60-mm Petri dishes (Falcon; Becton-Dickinson) in Eagle's medium essential medium (GIBCO Bio-Cult) supplemented with 10% fetal calf serum (GIBCO Bio-Cult), 2 mM L-glutamine, and 0.075% $NaHCO_3$. HeLa (human epithelial cervix carcinoma) cells were maintained under the same conditions as the Vero cells.

Viruses. The origin of the herpes simplex virus strains HSV-1 (KOS), HSV-2 (G), and TK^{-} HSV-1 (B2006) was as described previously (11).

Metabolism of [2-^{14}C]CEDU in Vero cells. Confluent Vero cells were either mock-infected or infected with HSV-1 (KOS), HSV-2 (G), or TK^{-} HSV-1 (B2006), at a multiplicity of infection of 0.01 PFU/cell. After a 1-hr incubation at 37°, mock medium or residual virus was removed, and cells were incubated for an additional 6 hr in the presence of [2-^{14}C]CEDU at a final concentration of 10 μM. Cells were washed three times with ice-cold phosphate-buffered saline and lysed with 200 μl of 0.2% sodium dodecyl sulfate, 0.5% *N*-laurylsarcosylate, 1 mM EDTA, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4 (lysis buffer). To precipitate acid-insoluble material, perchloric acid at a final concentration of 1 N was added to 100 μl of all samples. After a 10-min incubation on ice, samples were centrifuged in an Eppendorf microcentrifuge, and the supernatants were neutralized with K_2CO_3. Salt was removed by centrifugation, and 20-μl aliquots of the samples were spotted on silica gel thin layer plates (Merck 60 F254) and chromatographed in 1-butanol/acetic acid/water (2.5:1:1), 1-propanol/water (7:3), or FINK 8 [upper phase from a mixture of ethyl acetate/water/formic acid (40:15:5)] (12). After drying, the plates were cut into 0.5-cm pieces, and radioactivity was determined. Identification of the metabolites was possible by comparison of their R_F values with those of the co-migrating reference compounds (CEDU, CEU, and CEDUTP). Additionally, phosphorylated products could be detected by treating an aliquot of the acid-soluble fraction with 48 units of calf intestinal alkaline phosphatase (Boehringer Mannheim GmbH) in 50 mM Tris·HCl, pH 8.0. Samples were incubated at 37° for 30 min, and remaining enzyme was precipitated with perchloric acid at a final concentration of 1 N. After centrifugation, the supernatants were neutralized and chromatographed as described above. Proper controls were run in parallel to rule out possible breakdown of the metabolites during the processing of the samples.

Virus yield reduction. Confluent Vero cells in 60-mm Petri dishes were infected with HSV-1 (KOS), at a multiplicity of infection of 1 PFU/cell, in 0.5 ml Eagle's minimum essential medium (GIBCO) supplemented with 3% fetal calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. After a 1-hr adsorption period at 37°, residual virus was removed and 2 ml of medium containing 0, 0.5, 1, 5, 10, or 50 μM CEDU were added. Cells were incubated for an additional 24 hr at 37°, freeze-thawed once, and scraped off. Cell debris was removed by centrifugation. The supernatant was collected and stored at −70°. To determine the virus titer, the samples were thawed and diluted, and 0.5-ml volumes were applied onto confuent Vero cells in 60-mm Petri dishes. After a 1-hr incubation at 37°, virus was immobilized by an overlay of Earle's lactalbumin supplemented with 0.7% melted indubiose, 3% fetal calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. When the plaques had developed (usually after 2–3 days), the cells were covered with the same overlay now containing 0.017% neutral red. The number of plaques was recorded, and the virus titers of the cell culture supernatants were determined as PFU/ml.

Inhibition of viral DNA synthesis. Confluent Vero cells in 60-mm Petri dishes were infected with HSV-1 (KOS) at a multiplicity of infection of 1 PFU/cell. After a 1-hr adsorption period, residual virus was removed, and the cells were washed three times with phosphate-free Eagle's medium (GIBCO) and further incubated in the presence of varying concentrations of CEDU (0, 0.5, 1, 5, 10, or 50 μM) and a fixed concentration of [^{32}P]orthophosphate (250 μCi/Petri dish). After 24 hr, cells were lysed as described above, and the lysates were layered on top of 8 ml of a CsCl solution (1.72 g/ml). The gradients were centrifuged for 64 hr at 40,000 rpm in a TFT 65.13 Kontrol rotor. Seven-drop fractions were collected from the bottom of the tubes, and the refraction index of every fifth fraction was determined. The acid-insoluble material of the fractions was precipitated on Whatman GF/C filters with 5% ice-cold trichloroacetic acid. Filters were dried with ethanol, transferred to a toluene-based scintillant, and assayed for radioactivity in a liquid scintillation counter (Packard Tri-Carb 2660, with automatic correction for quenching).

Incorporation of [2-^{14}C]CEDU into DNA. Confluent Vero cells in 60-mm Petri dishes were infected with HSV-1 (KOS) at a multiplicity of infection of 1 PFU/cell. After a 1-hr adsorption period, residual virus was removed and the cells were incubated for an additional 24 hr in the presence of [2-^{14}C]CEDU at 0.5, 1, 5, 10, or 50 μM. The cells were then lysed and processed for CsCl equilibrium gradient analysis as described above. To determine in which form [2-^{14}C]CEDU was actually incorporated into DNA, the cells that had been exposed to 5 μM [2-^{14}C]CEDU for 24 hr were taken up in 200 μl of lysis buffer, and perchloric acid was added at a final concentration of 1 N. The sample was kept on ice for 10 min, acid-insoluble material was pelleted by centrifugation, and the pellet was washed twice with 1 N perchloric acid. Finally, the pellet was incubated in 100 μl of 1 N perchloric acid for 16 hr at 56°. The sample was then neutralized as described above and analyzed by thin layer chromatography in FINK 8. The proper reference compounds (i.e., CEDU, CEU, EDU, 5-ethyluracil, and 2′-deoxyuridine) were run in parallel.

DNA polymerase assays. Viral DNA polymerase was isolated from HSV-1 (KOS)-infected HeLa cells according to the procedure described by Knopf (13), with the final step being affinity chromatography on DNA cellulose. Enzymatic activity was determined in 20 mM Tris·HCl, pH 7.5, containing 100 mM $(NH_4)_2SO_4$. 100 μM each of dATP, dGTP, and dCTP, various concentrations of [*methyl*-^{3}H]dTTP, 100 μM dithiothreitol, 3 mM $MgCl_2$, 500 μg/ml bovine serum albumin, and 100 μg/

ml activated calf thymus DNA. The latter was prepared according to the method described by Baril *et al.* (14). Cellular (calf thymus) DNA polymerase α (P-L Biochemicals) was assayed in the same reaction mixture as viral DNA polymerase, except that $(NH_4)_2SO_4$ was omitted. The DNA polymerase reactions were initiated by adding HSV-1 (KOS) DNA polymerase or cellular DNA polymerase α, at a final concentration of 35 and 14 units/ml, respectively; 1 unit is defined as the amount of enzyme that leads to the incorporation of 1 pmol of dTMP/min into acid-insoluble material under the above conditions (with all nucleotide triphosphates at 100 μM). After a 30-min incubation at 37°, 1 ml of 5% ice-cold trichloroacetic acid was added, and after 10 min on ice and samples were applied to Whatman GF/C filters. The filters were washed with 5% trichloroacetic acid and dried with ethanol, and the radioactivity was determined. At the nucleoside triphosphate concentrations used, the rate of DNA polymerization was linear during the 30-min incubation period.

The ability of EDUTP and CEDUTP to support HSV-1 (KOS) and cellular DNA synthesis was evaluated in the absence of dTTP as substrate. [8-^{3}H]dATP served as the radiolabeled substrate. All nucleoside triphosphates were present at 100 μM, and the enzymatic activity was expressed as a percentage of the activity obtained when dTTP was added instead of EDUTP or CEDUTP. The background activity, obtained in the absence of dTTP or its analogues (CEDUTP, EDUTP), was set at 0%.

Results

Metabolism of CEDU by the cells. When [2-^{14}C]CEDU was incubated at a concentration of 10 μM, i.e., a concentration that is antivirally active (Fig. 1), for 6 hr with mock-infected, TK$^-$ HSV-1-infected, HSV-1 (KOS)-infected, or HSV-2 (G)-infected Vero cells, similar amounts of the unphosphorylated product were recovered from the cell lysates (Table 1). However, [2-^{14}C]CEDUMP levels were significantly increased in the HSV-1 (KOS)- and HSV-2 (G)-infected cells, as compared with the TK$^-$ HSV-1- or mock-infected cells. Furthermore, higher amounts of [2-^{14}C]CEDUDP and [2-^{14}C]CEDUTP were detected in HSV-1-infected cells than HSV-2-infected cells (Table 1). Other metabolites, i.e., the free pyrimidine base CEU and the furanyl derivative 5-(2-deoxy-β-D-ribosyl)-2,3-dihydro-5*H*-furano[2,3-*d*]pyrimidin-6-one, were searched for but could not be detected under the conditions used.

Inhibition of virus multiplication. Within the concentration range of 0.5–50 μM, CEDU effected a concentration-dependent inhibition of HSV-1 (KOS) multiplication in Vero cells (Fig. 1). At 50 μM, the highest concentration tested, CEDU reduced virus yield by 4 $\log_{10}$.

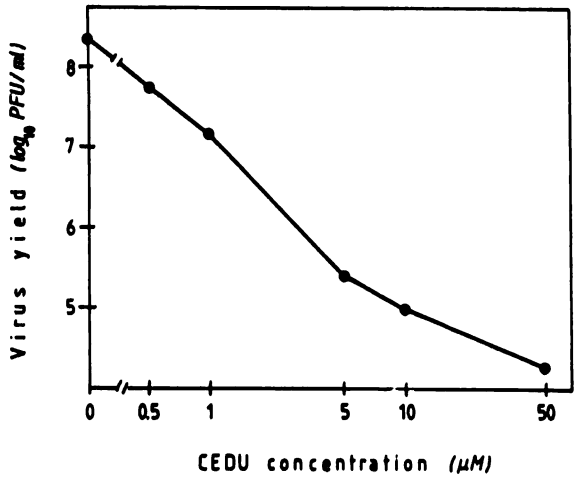

Fig. 1. Concentration-response effect of CEDU on HSV-1 (KOS) multiplication, based on the measurement of virus yield after incubation of HSV-1 (KOS)-infected Vero cells for 24 hr in the presence of varying concentrations of CEDU.

Inhibition of viral DNA synthesis. CEDU was evaluated for its inhibitory effect on viral DNA synthesis (monitored by the incorporation of [^{32}P]orthophosphate) under the same conditions as those employed for determining its inhibitory effect on virus multiplication. As shown in Fig. 2, viral DNA could be readily distinguished from cellular DNA by CsCl equilibrium gradient analysis. CEDU caused a concentration-dependent inhibition of both viral and cellular DNA synthesis. Inhibition of viral DNA synthesis was more pronounced than inhibition of cellular DNA synthesis. It was particularly apparent at CEDU concentrations of 5, 10, and 50 μM (Fig. 2, D–F).

Incorporation of CEDU into viral DNA. Incorporation of [2-^{14}C]CEDU into DNA of HSV-1 (KOS)-infected Vero cells was measured under the same conditions as those employed for determining its inhibitory effect on viral DNA synthesis. CsCl gradient analysis again allowed viral DNA-associated [2-^{14}C]CEDU to be distinguished from cellular DNA-associated [2-^{14}C]CEDU (Fig. 3). Incorporation of [2-^{14}C]CEDU into viral DNA was more pronounced than incorporation into cellular DNA; the preferential incorporation of [2-^{14}C]CEDU into viral DNA could be best demonstrated (Fig. 3D) at a CEDU concentration (i.e., 10 μM) that led to an equivalent synthesis of viral and cellular DNA (Fig. 2E).

The incorporation of [2-^{14}C]CEDU into viral DNA remained remarkably constant over a wide range of concentrations (0.5–50 μM) (Fig. 3). Although the concentration-response curve for the inhibition of viral DNA synthesis closely paralleled the concentration-response curve for virus yield reduction (Fig. 4), the concentration-response curve for CEDU incorporation into viral DNA followed an independent course. The fact that [2-^{14}C]CEDU incorporation into viral DNA remained high at CEDU concentrations (5, 10, and 50 μM) that were markedly inhibitory to viral DNA synthesis indicates that, as DNA synthesis decreased, incorporation of CEDU into DNA was actually facilitated.

The ^{14}C radioactivity associated with DNA of HSV-1 (KOS)-infected cells that had been exposed to [2-^{14}C]CEDU could unequivocally be ascribed to the incorporation of [2-^{14}C]CEDU; when the acid-insoluble material from such cells was subjected to acid hydrolysis, the only radiolabeled product detectable was [2-^{14}C]CEU (data not shown).

Inhibitory effects of DNA polymerase activity. In our previous study (15), EDU was found to achieve a concentration-dependent inhibition of HSV-1 (KOS) DNA synthesis and virus yield, quite similar to the concentration-dependent inhibition of viral DNA synthesis and yield by CEDU (Fig. 4). The 5′-triphosphates of EDU and CEDU were investigated in this study for their inhibitory effects on the activities of both purified HSV-1 (KOS) DNA polymerase (Fig. 5) and purified cellular DNA polymerase α (Fig. 6). From the Lineweaver-Burk plots, it appears that both EDUTP and CEDUTP caused a competitive inhibition of both viral and cellular DNA polymerase activity. K_i values (*s* being derived from *slope*) (16) were obtained from the secondary plots of the slopes versus inhibitory concentrations of EDUTP and CEDUTP (*inserts* in Figs. 5 and 6) (17). These values are presented in Table 2. The kinetic constants of dTTP, EDUTP, and CEDUTP are pre-

TABLE 1

Metabolism of [2-^{14}C]CEDU by virus-infected and uninfected Vero cells

Thin layer chromatographic analysis of acid-soluble material from either mock-infected, TK$^-$ HSV-1 (B2006)-infected, HSV-1 (KOS)-infected, or HSV-2 (G)-infected Vero cells exposed for 6 hr to [2-^{14}C]CEDU at 10 μM was performed. The solvent system used [1-butanol/acetic acid/water (2.5:1:1)] does not allow separation of the 5′-di- and 5′-triphosphates of CEDU. Data of a representative experiment are shown. Similar results were obtained in at least three independent experiments.

Vero cells	Intracellular concentration		
	[2-^{14}C]CEDU	[2-^{14}C]CEDUMP	[2-^{14}C]CEDUDP + [2-^{14}C]CEDUTP
		pmol/10^6 cells	
Mock-infected	22	10	2
TK$^-$ HSV-1 (B2006)-infected	23	11	1
HSV-1 (KOS)-infected	22	43	30
HSV-2 (G)-infected	21	45	10

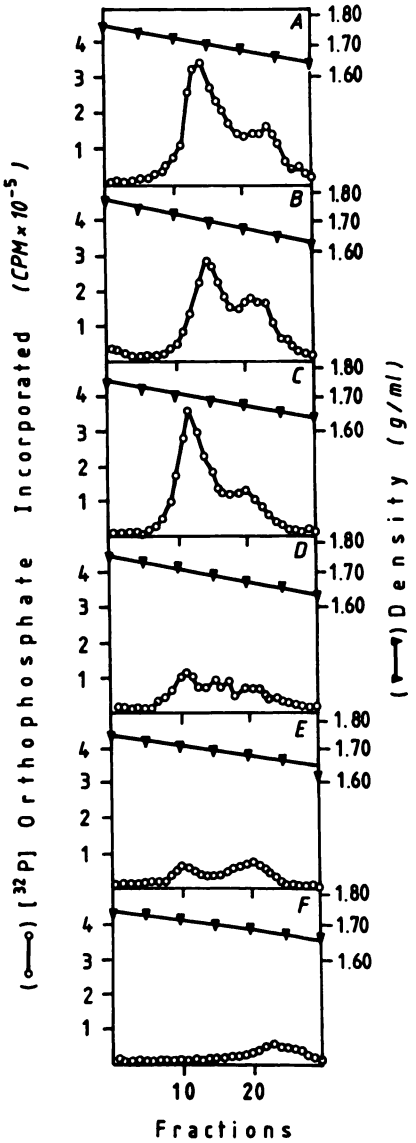

Fig. 2. CsCl equilibrium gradient analysis of DNA from HSV-1 (KOS)-infected Vero cells exposed for 24 hr to a fixed concentration of [^{32}P] orthophosphate (250 μCi/Petri dish) and varying concentrations of CEDU, 0 (A), 0.5 (B), 1 (C), 5 (D), 10 (E), or 50 μM (F). Data of a representative experiment are shown. Similar results were obtained in at least three independent experiments.

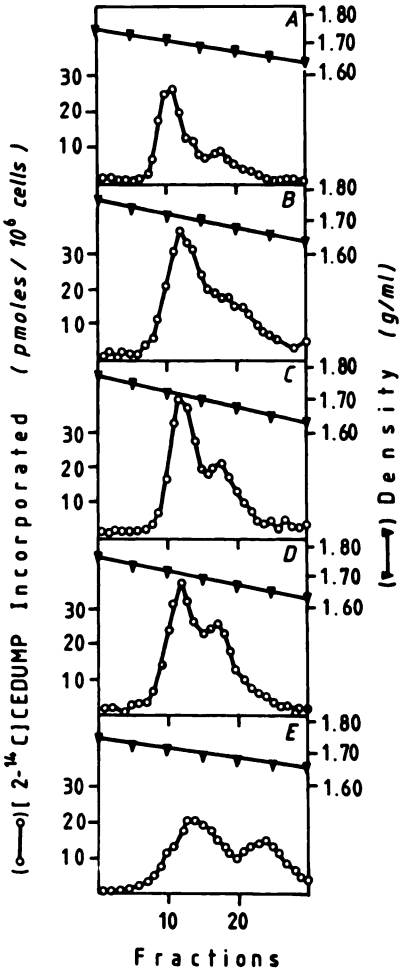

Fig. 3. CsCl equilibrium gradient analysis of DNA from HSV-1 (KOS)-infected Vero cells exposed for 24 hr to varying concentrations of [2-^{14}C]CEDU, 0.5 (A), 1 (B), 5 (C), 10 (D), or 50 μM (E). Data of a representative experiment are shown. Similar results were obtained in at least three independent experiments.

sented in Table 2. From these data, CEDUTP appears to be more potent as an inhibitor of DNA polymerase activity than EDUTP; CEDUTP and EDUTP are more inhibitory to the activity of HSV-1 (KOS) DNA polymerase than to the activity of cellular DNA polymerase α.

Alternative substrate activity for DNA polymerase. Not only were EDUTP and CEDUTP inhibitory to HSV-1 (KOS) DNA polymerase and cellular DNA polymerase α but, in the absence of the natural substrate dTTP, they also supported DNA synthesis. With DNA polymerase α the activity

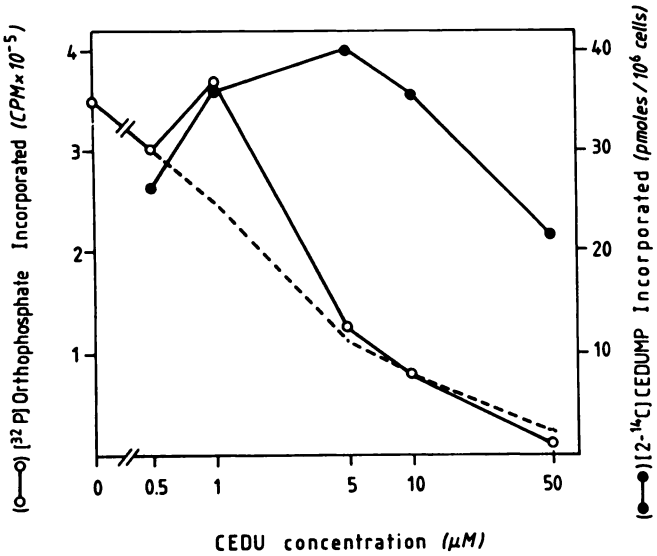

Fig. 4. Concentration-response curves for the inhibitory effect of CEDU on viral DNA synthesis (as monitored by the incorporation of [^{32}P] orthophosphate) and for incorporation of [2-^{14}C]CEDU into viral DNA. For this comparative evaluation, the peak values (for viral DNA) were taken from Figs. 2 and 3. – – –, Concentration-response curve for the inhibitory effect of CEDU on virus yield (Fig. 1).

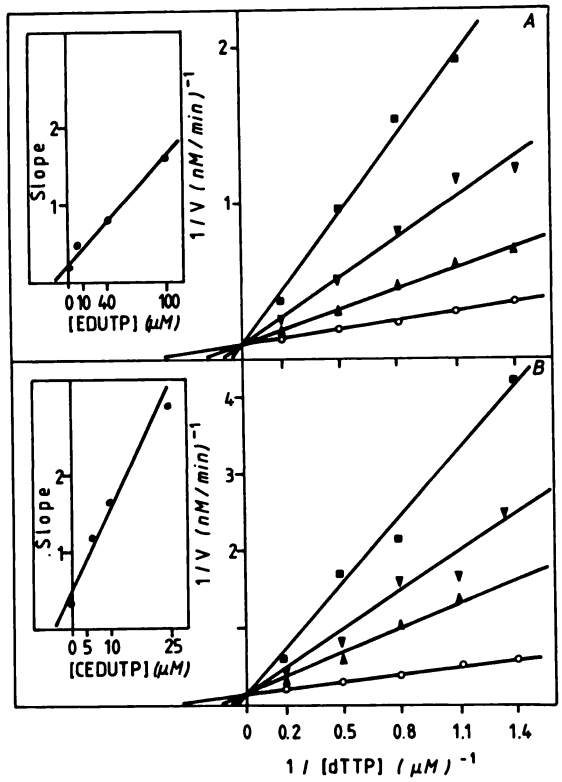

Fig. 5. Inhibition of HSV-1 (KOS) DNA polymerase by EDUTP (A) and CEDUTP (B). Inhibitory concentrations: O, control, ▲, 10 μM; ▼, 40 μM; and ■, 100 μM for EDUTP (A) and O, control; ▲, 5 μM; ▼, 10 μM; and ■, 25 μM for CEDUTP (B). Activated calf thymus DNA served as the template. The data are presented as Lineweaver-Burk plots with dTTP as the variable substrate. K_i values were determined by replotting the slopes versus the inhibitor concentrations, as shown in the *insets*.

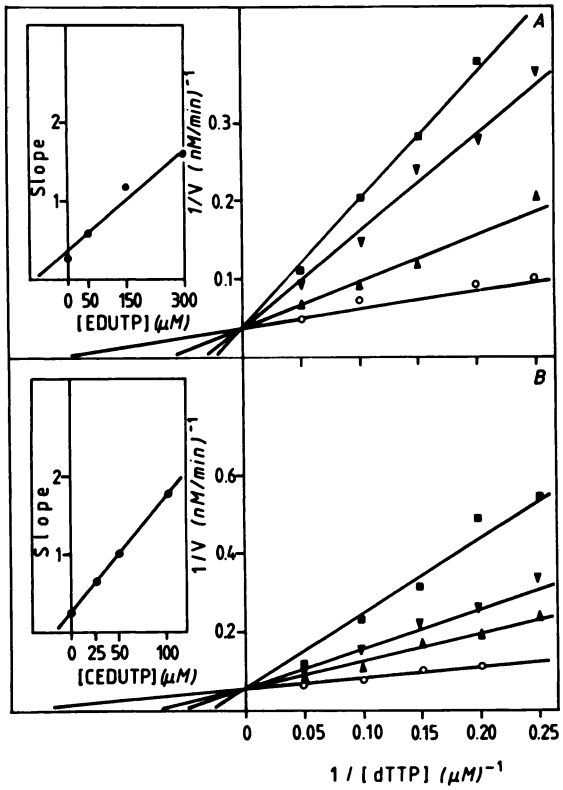

Fig. 6. Inhibition of calf thymus DNA polymerase α by EDUTP (A) and CEDUTP (B). Inhibitor concentrations: O, control; ▲, 50 μM; ▼, 150 μM; and ■, 300 μM for EDUTP (A) and O, control; ▲, 25 μM; ▼, 50 μM; and ■, 100 μM for CEDUTP (B). Activated calf thymus DNA served as the template. The data are presented as Lineweaver-Burk plots with dTTP as the variable substrate. K_i values were determined as explained in the legend to Fig. 5.

TABLE 2

Apparent kinetic constants of dTTP, EDUTP, and CEDUTP for viral and cellular DNA polymerase

Data of a representative experiment are shown.

DNA polymerase	K_m, dTTP	K_i	
		EDUTP	CEDUTP
		μM	
HSV-1 (KOS)	2.0	18.0	4.8
α (calf thymus)	9.2	108.5	15.6

TABLE 3

Ability of EDUTP and CEDUTP to support DNA synthesis in the absence of dTTP

Data of a representative experiment are shown.

Substrate (100 μM) added to reaction mixture	DNA synthesis	
	HSV-1 (KOS) DNA polymerase	DNA polymerase α (calf thymus)
	%	
dTTP	100	100
EDUTP	138	56
CEDUTP	112	52
None	0	0

was only partially restored, but with the viral DNA polymerase EDUTP and CEDUTP acted as even better substrates than dTTP (Table 3). If an inhibitory also acts as an alternative substrate for the enzymatic reaction, K_i values (Table 2) may

be considered as a measure of the affinity of the inhibitor for the enzyme (16), which implies that CEDUTP has a greater affinity for HSV-1 DNA polymerase than for cellular DNA polymerase α and that both enzymes have a greater affinity for CEDUTP than for EDUTP.

Discussion

In its mechanism of action and metabolism by the cells, CEDU behaves quite similarly to other 5-substituted 2′-deoxyuridine analogues such as EDU (15), the (*E*)-5-(2-halovinyl)-2′-deoxyuridines BVDU and IVDU (18), and the carbocyclic derivatives thereof (19). The compounds are preferentially phosphorylated by HSV-1-infected cells, they are incorporated into DNA of HSV-1-infected cells under conditions where they are not incorporated into DNA of uninfected cells, and within the infected cells they are incorporated to a greater extent into viral DNA than cellular DNA. In addition, the 5′-triphosphates of the 5-substituted 2′-deoxyuridine analogues are known to inhibit the activity of (viral and cellular) DNA polymerases competitively with respect to the natural substrate dTTP, as has been particularly shown for BVDUTP (20, 21). This inhibitory effect on the viral DNA polymerization reaction, together with the incorporation of the compounds into viral DNA, may account for their inhibitory effects on viral progeny formation.

The higher levels of CEDUMP achieved in HSV-infected cells, as compared to mock- or TK^- HSV-infected cells (Table 1), can obviously be ascribed to a preferential phosphorylation of CEDU by the virus-encoded TK. Similar observations have been made previously with EDU (15) and IVDU (22). Apparently, these thymidine analogues function as better substrates for the viral TK than for the cellular TK. The HSV-1-encoded, but not HSV-2-encoded, TK is also endowed with dTMP kinase activity (23, 24). This enzyme also recognizes BVDU 5′-monophosphate as substrate (25), and it must also be able to recognize the 5′-monophosphates of EDU and CEDU as substrates because they are more efficiently phosphorylated to the 5′-di- and 5′-triphosphates in HSV-1-infected cells than in HSV-2-infected cells.

In its 5′-triphosphate form, CEDU can act as both inhibitor and alternative substrate of viral and cellular DNA polymerases (Tables 2 and 3). CEDUTP is an even better substrate for HSV-1 DNA polymerase than for cellular DNA polymerase α. EDUTP is also a better substrate for viral DNA polymerase than for cellular DNA polymerase α (Table 2), which is consistent with previous observations (26). Akin to CEDUTP and EDUTP, other dTTP analogues, i.e., the triphosphates of BVDU and IVDU, are also able to act as alternative substrates of both viral and cellular DNA polymerases (27).

The propensity of CEDUTP to function as both inhibitor and substrate of the DNA polymerization reaction was reflected by its behavior in the cells, where CEDU was found to suppress viral DNA synthesis concomitantly with its incorporation into viral DNA. The inhibitory effect of CEDU on viral DNA synthesis showed a concentration dependence similar to that for its inhibitory effect on virus progeny formation (Fig. 4), suggesting a causal relationship between these two phenomena.

Based on the present study, the mechanism of action of CEDU could be summarized as follows (Fig. 7). Its selectivity as an antiherpes agent primarily depends on a preferential phosphorylation by the viral thymidine/dTMP kinase enzyme, which converts the compound successively to CEDU mono- and CEDU diphosphate. Upon further phosphorylation to CEDUTP, the latter either can be incorporated (as CEDUMP) into viral DNA or can competitively inhibit the incorporation of dTTP. As a result, viral DNA synthesis is shut off and viral progeny formation is suppressed.

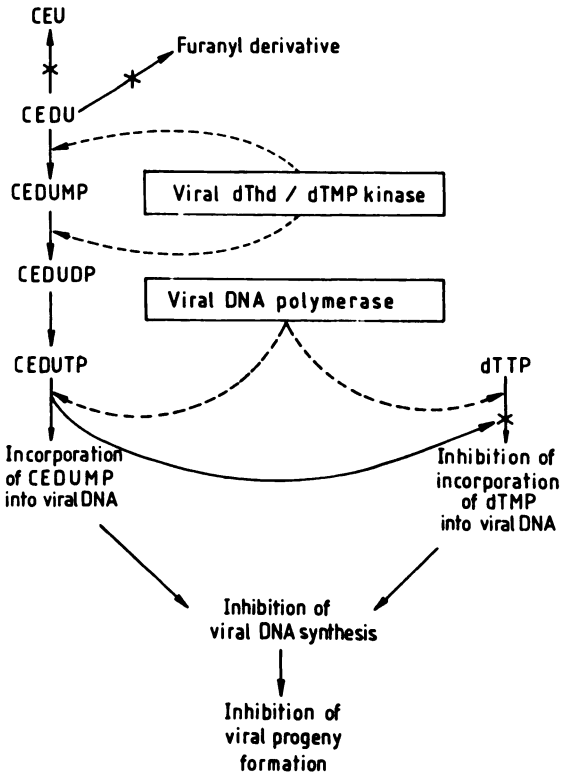

Fig. 7. Schematic presentation of mechanism of action of CEDU, as emerging from the present study. HSV-1 thymidine/dTMP kinase acts as the activating enzyme, preferentially confining further action of the compound to the virus-infected cell. The viral DNA polymerase then serves as the target enzyme for the active form of CEDU (CEDUTP). There is no evidence that Vero cells would convert CEDU to its free base (CEU) or 2,3-dihydro-5*H*-furano[2,3-*d*]pyrimidin-6-one derivative.

Which factor is directly responsible for the inhibition of viral DNA synthesis, the incorporation of CEDUMP into viral DNA or the competitive inhibitory effect of CEDUTP on the viral DNA polymerase enzyme? The inhibitory effect of CEDUTP on the viral DNA polymerase reaction (Fig. 5) was achieved at concentrations (5, 10, and 25 μM) that were fully inhibitory to viral DNA synthesis (Fig. 4) and viral progeny formation (Fig. 1). Therefore, it is tempting to attribute the inhibitory effect of CEDU on viral DNA synthesis to inhibition of the DNA polymerization reaction by CEDUTP.

However, CEDUTP also serves as an alternative substrate of DNA polymerase and, taking into account that with increasing concentrations of CEDU reduced amounts of DNA are synthesized, CEDU is more efficiently incorporated into DNA as its concentration increases. For other thymidine analogues, i.e., EDU (15) and BVDU (28), the incorporation into viral DNA increases proportionally with their suppressive effects on viral DNA synthesis and viral progeny formation. For EDU, the following sequence of events has been proposed: (i) incorporation into viral DNA, (ii) inhibition of viral DNA synthesis, and (iii) cessation of viral progeny formation. For CEDU, it is not clear to what extent its incorporation into viral DNA contributes to its inhibitory effect on viral DNA synthesis. It

depends on how efficiently the DNA that has incorporated CEDU acts as template for the next round of DNA replication. This is an issue that requires further investigation.

Acknowledgments

We thank Ivan Votruba for valuable advice, Ria Van Berwaer for excellent technical help, and Christiane Callebaut and Laurent Palmaerts for fine editorial help.

References

1. De Clercq, E. Synthetic pyrimidine nucleoside analogues. in *Approaches to Antiviral Agents* (M. R. Harden, ed.). MacMillan Press, London, 57–99 (1985).
2. De Clercq, E. Towards a selective chemotherapy of virus infections: development of bromovinyldeoxyuridine as a highly potent and selective antiherpetic drug. *Verh. K. Acad. Geneeskd. Belg.* **48**:261–290 (1986).
3. Griengl, H., M. Bodenteich, W. Hayden, E. Wanek, W. Streicher, P. Stütz, H. Bachmayer, I. Ghazzouli, and B. Rosenwirth. 5-(Haloalkyl)-2′-deoxyuridine: a novel type of potent antiviral nucleoside analogue. *J. Med. Chem.* **28**:1679–1684 (1985).
4. De Clercq, E., and B. Rosenwirth. Selective *in vitro* and *in vivo* activities of 5-(2-haloalkyl)pyrimidine nucleoside analogs, particularly 5-(2-chloroethyl)-2′-deoxyuridine, against herpes simplex virus. *Antimicrob. Agents Chemother.* **28**:246–251 (1985).
5. Maudgal, P. C., and E. De Clercq. Evaluation of bromovinyldeoxyuridine-related compounds in the treatment of experimental herpes simplex keratitis. *Arch. Ophthalmol.* **103**:1393–1397 (1985).
6. Maudgal, P. C., E. De Clercq, R. Bernaerts, M. Dieltiens, M. Breemersch, and L. Van Eeckhoutte. Ocular penetration and efficacy of chloroethyldeoxyuridine against herpetic keratouveitis. *Invest. Ophthalmol. Visual Sci* **27**:1453–1458 (1986).
7. De Clercq, E. Virus-drug resistance: thymidine kinase-deficient (TK^-) mutants of herpes simplex virus: therapeutic approaches. *Ann. Ist. Super. Sanità* **23**:841–848 (1987).
8. Desgranges, C., E. De Clercq, G. Razaka, F. Drouillet, I. Belloc, and H. Bricaud. Deoxyribosyl exchange reactions leading to the *in vivo* generation and regeneration of the antiviral agents (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, 5-ethyl-2′-deoxyuridine and 5-(2-chloroethyl)-2′-deoxyuridine. *Biochem. Pharmacol.* **35**:1647–1653 (1986).
9. Yoshikawa, M., T. Kato, and T. Takenishi. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* 5065–5068 (1967).
10. Hoard, D. E., and D. G. Ott. Conversion of mono- and oligodeoxyribonucleotides to 5′-triphosphates. *J. Am. Chem. Soc.* **87**:1785–1788 (1965).
11. De Clercq, E., J. Descamps, G. Verhelst, R. T. Walker, A. S. Jones, P. F. Torrence, and D. Shugar. Comparative efficacy of different antiherpes drugs against different strains of herpes simplex virus. *J. Infect. Dis.* **141**:563–574 (1980).
12. Fink, K., R. E. Cline, R. B. Henderson, and R. M. Fink. Metabolism of thymine (*methyl*-C^{14} or -2-C^{14}) by rat liver *in vitro*. *J. Biol. Chem.* **221**:425–433 (1956).
13. Knopf, K.-W. Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. *Eur. J. Biochem.* **98**:231–244 (1979).
14. Baril, E., J. Mitchener, L. Lee, and B. Baril. Action of pancreatic DNase: requirements for activation of DNA as a template-primer for DNA polymerase α. *Nucleic Acids Res.* **4**:2641–2653 (1977).
15. De Clercq, E., and R. Bernaerts. Specific phosphorylation of 5-ethyl-2′-deoxyuridine by herpes simplex virus-infected cells and incorporation into viral DNA. *J. Biol. Chem.* **262**:14905–14911 (1987).
16. Spector, T., and W. W. Cleland. Meanings of K_i for conventional and alternate-substrate inhibitors. *Biochem. Pharmacol.* **30**:1–7 (1981).
17. Segel, I. H. *Biochemical Calculations.* J. Wiley & Sons, Inc., New York, (1976).
18. De Clercq, E., and R. T. Walker. Synthesis and antiviral properties of 5-vinylpyrimidine nucleoside analogs. *Pharmacol. Ther.* **26**:1–44 (1984).
19. De Clercq, E., R. Bernaerts, J. Balzarini, P. Herdewijn, and A. Verbruggen. Metabolism of the carbocyclic analogue of (*E*)-5-(2-iodovinyl)-2′-deoxyuridine in herpes simplex virus-infected cells: incorporation of C-IVDU into DNA. *J. Biol. Chem.* **260**:10621–10628 (1985).
20. Allaudeen, H. S., J. W. Kozarich, J. R. Bertino, and E. De Clercq. On the mechanism of selective inhibition of herpesvirus replication by (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. *Proc. Natl. Acad. Sci. USA* **78**:2698–2702 (1981).
21. Ruth, J. L., and Y.-C. Cheng. Nucleoside analogues with clinical potential in antivirus chemotherapy. *Mol. Pharmacol.* **20**:415–422 (1981).
22. Descamps, J., and E. De Clercq. Specific phosphorylation of *E*-5-(2-iodovinyl)-2′-deoxyuridine by herpes simplex virus-infected cells. *J. Biol. Chem.* **256**:5973–5976 (1981).
23. Chen, M. S., and W. H. Prusoff. Association of thymidylate kinase activity with pyrimidine deoxyribonucleoside kinase induced by herpes simplex virus. *J. Biol. Chem.* **253**:1325–1327 (1978).
24. Chen, M. S., W. P. Summers, J. Walker, W. C. Summers, and W. H. Prusoff. Characterization of pyrimidine deoxyribonucleoside kinase (thymidine kinase) and thymidylate kinase as a multifunctional enzyme in cells transformed by herpes simplex virus type 1 and in cells infected with mutant strains of herpes simplex virus. *J. Virol.* **30**:942–945 (1979).
25. Fyfe, J. A. Differential phosphorylation of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine monophosphate by thymidylate kinases from herpes simplex viruses types 1 and 2 and varicella zoster virus. *Mol. Pharmacol.* **21**:432–437 (1982).
26. Kowalzick, L., K. K. Gauri, S. Spadari, G. Pedrali-Noy, J. Kühne, and G. Koch. Differential incorporation of thymidylate analogues into DNA by DNA polymerase α and by DNA polymerases specified by two herpes simplex viruses. *J. Gen. Virol.* **62**:29–38 (1982).
27. Allaudeen, H. S., M. S. Chen, J. J. Lee, E. De Clercq, and W. H. Prusoff. Incorporation of *E*-5-(2-halovinyl)-2′-deoxyuridines into deoxyribonucleic acids of herpes simplex virus type 1-infected cells. *J. Biol. Chem.* **257**:603–606 (1982).
28. Mancini, W. R., E. De Clercq, and W. H. Prusoff. The relationship between incorporation of *E*-5-(2-bromovinyl)-2′-deoxyuridine into herpes simplex virus type 1 DNA with virus infectivity and DNA integrity. *J. Biol. Chem.* **258**:792–795 (1983).

Send reprint requests to: Prof. E. De Clercq, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.