Specific Phosphorylation of 5-Ethyl-2'-deoxyuridine by Herpes Simplex Virus-infected Cells and Incorporation into Viral DNA*

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5-Ethyl-2'-deoxyuridine (EDU) is a potent and selective inhibitor of the replication of herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), which is currently being pursued for the topical treatment of HSV-1 and HSV-2 infections in humans. Using [4-14C]EDU as the radiolabeled analogue of EDU, it was ascertained that, at antivirally active doses, EDU is phosphorylated to a much greater extent by HSV-infected Vero cells and the by mock-infected cells. Within the HSV-1-infected cells, EDU was incorporated to a much greater extent into viral DNA than cellular DNA. Using varying doses of EDU, a close correlation was found between the incorporation of EDU into viral DNA, the inhibition of viral DNA synthesis, and the inhibition of virus yield. It is postulated that the selectivity of EDU as an antiviral agent depends on both its preferential phosphorylation by the virus-infected cell and its preferential incorporation into viral DNA. The latter then results in a suppression of viral DNA synthesis and, hence, shutoff of viral progeny formation.

Among the varying antiviral compounds which have been described in the past two decades, the 5-substituted 2'-deoxyuridine 5-ethyl-2'-deoxyuridine (EDU) (1, 2) takes a place apart. Its chemotherapeutic potential for the treatment of herpesvirus infections was first mentioned in 1967 by Gauri and Malorny (3) and in 1968 by Gauri (4). The synthesis of EDU was reported by Swierkowski and Shugar (5), who also emphasized the absence of mutagenic activity of the compound. This contrasts with the earlier developed anti-herpes agents 5-iodo-2'-deoxyuridine and 5-trifluoro-2'-deoxythymidine which have been in clinical use for almost 25 years (for the topical treatment of herpetic eye infections) despite their toxicities and adverse effects (6). That EDU is a relatively innocuous compound is further attested by its failure to induce chromosomal aberrations (7, 8) and sister chromatid exchange (6) in human lymphocytes or fibroblasts and its failure to induce the release of retrovirus particles from cell lines such as murine BALB/3T3 cells (9, 10). This again contrasts with 5-iodo-2'-deoxyuridine, which is notorious for its inductive effect on the production of retrovirus particles (10).

In spite of the lack of any solid antiviral data in animal models, EDU has been pursued for its clinical potential in the topical treatment of herpetic keratitis (11) and herpetic skin infections (12). The results of these clinical trials were inconclusive. More recently, EDU has been the subject of extensive investigations in animal models, and these studies revealed that EDU is as effective as, if not more so than, acyclovir in the topical treatment of mucocutaneous HSV-1 and HSV-2 infections in guinea pigs (13, 14). Controlled double-blind clinical trials have been initiated to assess the value of EDU in the topical treatment of genital herpes in humans.2

Very little is known about the mechanism of action of EDU. The compound has been found effective against various laboratory strains and clinical isolates of HSV-1 and HSV-2 at a minimum effective dose varying within the range of 1–10 μM, depending on the cell system used (14–16). EDU is inactive against thymidine kinase-deficient mutants of HSV-1 (TK−HSV-1) (14, 15, 17), which suggests that its antiviral activity must be mediated by the virus-encoded thymidine kinase; and, in fact, EDU has a much higher affinity for both the HSV-1- and HSV-2-encoded thymidine kinases than for the host (HeLa) cell (cytoplasmic and mitochondrial) enzymes (18). How these findings relate to the selective inhibitory effect of EDU on HSV replication has never been clarified, however.

These studies were designed to obtain better insight in the mode of antiviral action of EDU. Therefore, the metabolism of EDU was followed in both uninfected and virus-infected cells using radiolabeled [4-14C]EDU or [2-14C]EDU to monitor the metabolic conversions of the compound. The final target for the antiviral action of EDU was identified as viral DNA. EDU was preferentially incorporated into viral DNA, and this incorporation then resulted in a specific inhibition of viral DNA synthesis concomitantly with a reduction in virus yield.

MATERIALS AND METHODS

Compounds—EDU and [2-14C]EDU were kindly provided by Dr. E. Mauz and Dr. B. Hempel (Robugen GmbH Pharmazeutische Fabrik, Easinglen/Neckar, Federal Republic of Germany); [2-14C] EDU had a specific activity of 12 mCi/mmol. [4-14C]EDU was obtained from Dr. D. Ilse (Ortho Pharmaceutical Ltd., Don Mills, Ontario, Canada). The compound had a specific activity of 54 mCi/mmol.

Cells—Vero cells were grown to confluency in Falcon 60-mm Petri dishes (1 x 106 cells) in Eagle's minimum essential medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine (GIBCO), and 0.075% NaHCO3 (GIBCO).

2 D. Ilse, personal communication.
Viruses—The origin of the herpes simplex virus strains HSV-1 (strain KOS), HSV-2 (strain G), and TK-HSV-1 (strain B2006) has been described previously (15).

Metabolism of [4-14C]EDU in Vero Cells—Confluent Vero cells were either mock-infected or infected with HSV-1 (KOS), HSV-2 (G), and TK-HSV-1 (strain B2006) at a multiplicity of infection of 300 pfu/cell. After a 1-h incubation at 37 °C, 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-laurylsarcosine, 1 mM EDTA, 0.1 N NaCl, and 10 mM Tris-HCl, pH 7.4.

To precipitate acid-insoluble material, perchloric acid at a final concentration of 1 N was added to 100 µl of each sample, and the pellets were washed with 0.1 N perchloric acid and incubated overnight at 56 °C. After centrifugation, the supernatants were neutralized with K2CO3 and chromatographed on thin-layer plates (see above) in FINK8 with EDU, EDUMP, EU, and uracil as reference compounds. After drying, the plates were cut into 0.5-cm pieces, and radioactivity was determined.

Salt was removed by centrifugation at 0 °C with perchloric acid at a final concentration of 1 N. The pellets were then resuspended in 50 µl of 1 N NaCl, and the supernatants were neutralized with K2CO3 and chromatographed in 1-butanol/acetic acid/water (2.5:1:1) or 1-propanol/water (70:30) or FINK8 (19). After drying, the plates were cut into pieces of 0.5 cm, and radioactivity was determined.

To identify the metabolites, Rf values of the radioactivity peaks were compared to Rf values of the reference compounds EDU, its 5'-monophosphate EDUMP, and 5-ethyluracil (EU). To identify phosphorylated compounds, 20 µl of the acid-soluble fraction was treated with 48 units of calf intestinal alkaline phosphatase (Boehringer Mannheim GmbH) in 50 mM Tris-HCl, pH 8.0. Samples were incubated for 30 min at 37 °C, and protein precipitates were precipitated on Whatman GF/C filters with 5% ice-cold trichloroacetic acid. Filters were dried with ethanol, and the radioactivity was determined.

To precipitate acid-insoluble material, perchloric acid at a final concentration of 1 N was added to 100 µl of each sample, and after a 10-min incubation on ice, samples were centrifuged in an Eppendorf microcentrifuge, and the supernatants were neutralized with K2CO3.

The acid-insoluble material derived from HSV-1 (KOS)-infected vero cells, incubated for 24 h in the presence of [4-14C]EDU at 5 µM, was washed three times with ice-cold perchloric acid. The pellet was then resuspended in 50 µl of 1 N perchloric acid and incubated overnight at 56 °C. After centrifugation, the supernatant was neutralized with K2CO3 and chromatographed on thin-layer plates (see above) in FINK8 with EDU, EDUMP, EU, and uracil as reference compounds. After drying, the plates were cut into 0.5-cm pieces, and radioactivity was determined.

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Metabolism of EDU by Mock- and HSV-infected Vero Cells—When [4-14C]EDU was incubated at a concentration of 5 µM (which is a dose that is antivirally active (Fig. 7A)) for 24 h with mock-infected or TK HSV-1-infected Vero cells, the main products recovered from the cell lysate were EDUMP, the 5'-monophosphate of EDU, and EDU itself (Fig. 1, A and B). As revealed by the amounts of EDUMP recovered from the cell lysates, EDU was phosphorylated to a much greater extent by the HSV-1 (KOS)- and HSV-2 (G)-infected cells than by the mock- and TK-HSV-1 (B2006)-infected cells (Fig. 1). The main phosphorylated product detected in the HSV-2 (G)-infected cell lysates was EDUMP, whereas the HSV-1 (KOS)-infected cell lysates predominantly yielded a higher phosphorylated product which could be identified as either the 5'-di- or 5'-triphosphate of EDU, or both. The solvent system used (1-butanol/acetic acid/water (2.5:1:1)) does not allow separation of the 5'-di- and 5'-triphosphates (20, 21).

That the slowly moving radioactive peaks corresponded to EDUDP/EDUTP was further ascertained by subjecting the acid-soluble material obtained from the HSV-1 (KOS)-infected Vero cells to treatment with alkaline phosphatase (Fig. 2). Upon such treatment, the TLC peaks identified as EDUMP and EDUDP/EDUTP disappeared completely, and the peak corresponding to EDU increased considerably, suggesting that the phosphorylated products had been converted back to their nucleoside form.

When [2-14C]EDU was incubated with the mock- or virus-infected cells at a concentration of 200 µM (which is far above the minimum antiviral concentration (Fig. 7A)), EDU was metabolized differently from what had been observed at a concentration of only 5 µM (Fig. 1). First, when exposed to this high EDU concentration, mock-, TK-HSV-1-, HSV-1 (KOS)-, and HSV-2 (G)-infected cells proved about equally effective in phosphorylating the compound to its 5'-monophosphate (Fig. 3); and second, an additional product, identified as EU, was found which had not been detected in cells exposed to 5 µM EDU. Thus, the preferential phosphorylation of EDU to EDUMP and further onto EDUDP/EDUTP which has been observed in HSV-1 (KOS)-infected Vero cells ex-
This material was either untreated calf intestinal alkaline phosphatase of the reference compounds EDUMP, EDU, and EU. 

infected Vero cells exposed for 24 h to 

\[4-^{14}C\]EDU at and HSV-2 (G)-infected compounds EDUMP, EDU, and EU. The position of EDUDP EDUTP was deduced based upon the results obtained for the experiments presented in Figs. 1 and 2.

Under conditions where [methyl-\(^3\)H]dThd was effectively incorporated into DNA of HSV-1 (KOS)-infected cells (Fig. 1C), one might infer that EDUTP in HSV-1 (KOS)-infected cells (Fig. 4C), [\(^4\)C]EDU was preferentially incorporated into viral DNA (Fig. 4D). The extent to which [\(^4\)C]EDU was incorporated into viral DNA of HSV-2 (G)-infected cells (Fig. 4F) was considerably lower than the extent to which [\(^4\)C]EDU was incorporated into HSV-1 (KOS) DNA (Fig. 4D) and so was the extent of incorporation of [methyl-\(^3\)H]dThd into HSV-2 (G) DNA (Fig. 4E) relative to its incorporation into HSV-1 (KOS) DNA (Fig. 4C). In globulo, the extent to which [\(^4\)C]EDU was incorporated into DNA of mock-, HSV-2 (G)-, and HSV-1 (KOS)-infected Vero cells (Fig. 4B, F, and D, respectively) reflected the phosphorylation pattern of [\(^4\)C]EDU by these cells, thus, in increasing order of phosphorylation: mock-infected cells < HSV-2 (G)-infected cells < HSV-1 (KOS)-infected cells (Fig. 1, A, D, and C, respectively).

A preferential incorporation of [\(^4\)C]EDU into viral DNA of HSV-1 (KOS)-infected Vero cells was noted at [\(^4\)C]EDU concentrations as low as 0.5 \(\mu\)M (Fig. 5B) and 1.0 \(\mu\)M (Fig. 5D). As the concentration of [\(^4\)C]EDU increased, its incorporation into cellular DNA increased concomitantly (Fig. 5B, D, F, and J), while its incorporation into viral DNA remained quasi constant over a broad concentration range (0.5-10 \(\mu\)M) and at 50 \(\mu\)M was almost totally suppressed. The fact that the incorporation of [\(^4\)C]EDU into HSV-1 (KOS) DNA did not increase with increasing concentrations of [\(^4\)C]EDU and actually decreased from a concentration of 10 \(\mu\)M appeared to be related to the inhibition of viral DNA synthesis that must have ensued from the incorporation of EDU. When viral and cellular DNA synthesis in HSV-1 (KOS)-infected cells was monitored based on the incorpora-
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Fig. 5. CsCl equilibrium gradient analysis of DNA from HSV1 (KOS)-infected Vero cells exposed for 24 h to either varying concentrations of [4-14C]EDU (B, D, F, H, J) or varying concentrations of EDU and a fixed concentration of [32P]orthophosphate (25 μCi/Petri dish) (A, C, E, G, I, K). The concentrations of [4-14C]EDU or EDU were 0 μM (A), 0.5 μM (B, C), 1 μM (D, E), 5 μM (F, G), 10 μM (H, I), and 50 μM (J, K).

The radioactive material incorporated into DNA of HSV-1 (KOS)-infected Vero cells which had been exposed to [4-14C]EDU really corresponded to EDU and not to any other radiolabeled products was ascertained by examining the acid hydrolysate of this DNA by TLC (Fig. 6). This analysis revealed three peaks, two of which could be identified as EU and EDUMP, respectively, based on the RF values of the corresponding markers. The third, nonmigrating peak must represent nondegraded material. Uracil was not detected in the DNA hydrolysate. The release of EU must have resulted from cleavage of the N-glycosidic linkage by the rather drastic acid treatment (1 N perchloric acid overnight at 56°C).

Fig. 6. TLC analysis of the acid hydrolysate of acid-precipitable material derived from HSV-1 (KOS)-infected Vero cells exposed for 24 h to [4-14C]EDU at 5 μM. Arrows indicate the positions of reference compounds EDUMP, EDU, EU, and uracil (U).

Fig. 7. Dose-dependent effect of EDU on HSV-1 (KOS) multiplication as based on the measurements of virus yield (A), viral DNA synthesis as monitored by [32P]orthophosphate incorporation into viral DNA recovered from CsCl gradients (B), and ratio of the amount of [4-14C] EDU (pmol/10⁶ cells) incorporated into viral DNA to the amount of viral DNA synthesized (cpm of [32P]orthophosphate incorporated), with both the ¹⁴C- and ³²P-labeled DNAs recovered from CsCl gradients (C).

Correlation between Incorporation of EDU into Viral DNA and Inhibition of Virus Replication—Within the dosage range used (1–50 μM), EDU caused a dose-dependent inhibition of HSV-1 (KOS) multiplication (Fig. 7A). At 5 μM, virus yield
was reduced by 1.3 log_{10}; and at 50 \mu M, it was reduced by more than 3 log_{10}. When the inhibition of viral DNA synthesis, as monitored by $^{32}$Porthophosphate incorporation was plotted in function of increasing concentrations of EDU (Fig. 7B), it showed a similar dose-dependent effect. Conversely, the incorporation of [4-14C]EDU into viral DNA relative to the amount of viral DNA that was synthesized increased almost proportionally with increasing EDU concentrations (Fig. 7C).

The data presented in Fig. 7 (A-C) pointed to a close correlation between the inhibitory effect of EDU on HSV-1 (KOS) replication, its inhibitory effect on viral DNA synthesis, and its incorporation into viral DNA. The correlation between these three parameters was further assessed by regression analysis (Fig. 8). The correlation coefficients found were: $r = 0.995$ for the correlation between the inhibition of virus yield and inhibition of viral DNA synthesis (Fig. 8A), $r = -0.944$ for the correlation between inhibition of virus yield and the relative amount of EDU incorporated into viral DNA (Fig. 8B), and $r = -0.955$ for the correlation between inhibition of viral DNA synthesis and the relative amount of EDU incorporated into viral DNA (Fig. 8C).

**DISCUSSION**

EDU was phosphorylated to a 50-fold greater extent by HSV-1- and HSV-2-infected cells than by either mock- or TK-HSV-1-infected cells (Fig. 1), which indicates that EDU is preferentially used as substrate by the virus-induced thymidine kinase. This is in line with the observations of Cheng (18) who found that EDU has a much higher affinity for HSV-1 and HSV-2 thymidine kinases than for HeLa cytoplasmic and mitochondrial thymidine kinases (apparent dissociation constants of 0.7, 0.3, 82, and 305 \mu M, respectively). The fact that EDUMP is readily phosphorylated onto EDUDP/EDUTP in HSV-1-infected, but much less so in HSV-2-infected, cells is consistent with the notion that the HSV-1-encoded, but not the HSV-2-encoded, thymidine kinase is endowed with thymidylate kinase activity (22, 23). Apparently, this dThd kinase-associated dTMP kinase must be able to recognize EDUMP as a substrate.

When the mock- or virus-infected Vero cells were exposed to a supraoptimal concentration (200 \mu M) of EDU, 5-ethyluracil was formed (Fig. 3). This product must have resulted from the phosphorolytic cleavage of the N-glycosidic linkage of EDU by pyrimidine-nucleoside phosphorylases such as thymidine phosphorylase (24, 25). EDU is as good a substrate for this enzyme, as are dUrd, dThd, and various other 5-substituted dUrd derivatives (24, 25). Conceivably, Vero cells contain sufficient phosphorylase activity to degrade EDU to EU, at least if the substrate is in excess, and this activity is independent of the virus infection.

EDUMP has been shown to inhibit thymidylate synthase from various sources (26, 27). However, compared to other 5-substituted dUMPs, such as 5-fluoro-dUMP, 5-trifluoromethyl-dUMP, 5-nitro-dUMP, and 5-formyl-dUMP (26, 28), EDUMP is a relatively poor inhibitor of dTMP synthase, i.e. its $K_i$ for murine L1210 leukemia and Ehrlich ascites carcinoma dTMP synthases is 35.6 and 56.1 \mu M, respectively (27), and these values are considerably higher than those at which EDU is antivirally active or detectably incorporated into viral DNA (0.5 \mu M (Fig. 5B)). The inhibitory effect of EDUMP on dTMP synthase may be responsible for the inhibition of tumor cell proliferation by EDU (28), but does not appear to account for its antiviral activity.

5-Alkyl-dUTPs, and particularly EDUTP, can serve as alternate substrates for mammalian, viral, and bacterial DNA polymerases (29–31). EDUTP is even a better substrate for HSV-1 and HSV-2 DNA polymerases than for the cellular DNA polymerases $\alpha$ and $\beta$, with its $K_i$ for the viral (HSV-2) enzyme of 1.06 \mu M as compared to 16 \mu M for the cellular (\beta) enzyme (30, 31). These observations apparently extend to the in vivo (cell culture) situation, where [4-14C]EDU was found to be more extensively incorporated into viral than cellular DNA (Fig. 5). Thus, EDUTP acts as a better substrate for the viral than for the cellular DNA polymerases both in vitro (cell-free enzyme) and in vivo (cell culture).

With increasing doses of EDU added to the cell culture medium, increasing amounts of EDU are incorporated into viral DNA; and the more EDU is incorporated, the more viral DNA synthesis is suppressed (Fig. 7). How the incorporation of EDU leads to an inhibition of viral DNA synthesis remains subject of further study. Presumably, DNA that has incorporated EDU is less efficient a template for further DNA replication than is normal DNA. In any case, the inhibition of viral DNA synthesis is paralleled by a reduction in virus yield, and all three events, incorporation of EDU into viral DNA,
inhibition of viral DNA synthesis, and reduction of virus progeny, are closely correlated with each other (Fig. 8). This may be indicative of the following sequence of events: (i) incorporation of EDU into viral DNA, (ii) inhibition of viral DNA synthesis, and (iii) shutoff of viral progeny formation.

The subsequent metabolic reactions involved in the antiviral action of EDU are summarized in Fig. 9. The selectivity of EDU as an anti-herpes agent depends on (i) a preferential phosphorylation by the virus-induced thymidine kinase and the herewith associated thymidylate kinase, and (ii) the preferential incorporation of EDUTP into viral DNA. The latter is then followed by a suppression of viral DNA synthesis and viral progeny formation. To allow EDU to achieve its selective inhibitory effect on virus replication, a number of enzymatic reactions should be avoided which would otherwise lead to a premature degradation of the compound (i.e. pyrimidine-nucleoside phosphorylase) or make the compound nocuous to the host cell (i.e. cellular thymidine and/or thymidylate kinase and cellular DNA polymerases). Host cell toxicity may result from either inhibition of thymidylate synthase or incorporation into cellular DNA, or both. Either event may lead to an inhibition of DNA synthesis and, consequently, cell proliferation; but, as shown in Fig. 5K, inhibition of cellular DNA synthesis by EDU is seen only at a concentration of 50 μM, as is the inhibition of dTMP synthase by EDUMP (27), and this concentration is far in excess of the minimum effective dose required to inhibit viral progeny formation (1 μM; see Fig. 7A).

The mechanism of action of EDU is comparable to that of the (E)-5-(2-halovinyl)-2'-deoxouridines BVDU and IVDU (32) in that they are (i) preferentially phosphorylated by the virus-encoded thymidine kinase, (ii) incorporated into DNA of HSV-1-infected cells under conditions where they are not incorporated into DNA of uninfected cells, and (iii) inhibit HSV-1-infected cells, preferentially incorporated into viral DNA. There are, however, a few (minor) differences between the behavior of EDU and the behavior of BVDU and IVDU. First, whereas [4-14C]EDU (Fig. 1) and [125I]IVDU (Figs. 1 and 2 of Ref. 21) were phosphorylated to the 5'-monophosphate to a 50-fold greater extent in HSV-1 (KOS)-infected cells than in mock-infected Vero cells, further phosphorylation to the 5'-di- and 5'-triphosphate forms occurred much more efficiently with EDU than with IVDU. Second, like EDUTP (29), IVDU and BVDU can partially substitute for dTTP in the DNA polymerase reactions (33), but whereas EDUTP is apparently a better substrate for the HSV-1 and HSV-2 DNA polymerases than for the cellular DNA polymerases α and β (30, 31), no marked differences were noted in the substrate activity of IVDU or BVDU for viral and cellular DNA polymerases (33); and in other experiments, BVDUTP was even found more inhibitory to HSV-1 DNA polymerase than to cellular DNA polymerases (34). Third, within the virus-infected cell, BVDU, IVDU, and EDU can be incorporated into both viral and cellular DNA. At supratherapeutic concentrations (≥0.5 μM for BVDU and IVDU (21, 33, 35) and ≥10 μM for EDU (Fig. 5)), the compounds seem to be equally well incorporated into HSV-1 DNA and host cell DNA. However, at doses ranging from 0.05 to 0.5 μM for BVDU and from 0.5 to 5 μM for EDU, either compound is preferentially incorporated into viral DNA; and, as established with BVDU previously (35) and EDU in this study, as more BVDU and EDU is incorporated into HSV-1 DNA, viral DNA synthesis and viral progeny formation are proportionally suppressed.

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