ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1985, p. 552–560
0066-4804/85/100552-09$02.00/0
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Vol. 28, No. 4

Antiviral Activity of 5-Ethyl-2′-Deoxyuridine against Herpes Simplex Viruses in Cell Culture, Mice, and Guinea Pigs

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Received 2 May 1985/Accepted 24 July 1985

The susceptibility of 3 laboratory strains and 24 clinical isolates of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) to 5-ethyl-2′-deoxyuridine was determined in plaque reduction assays in Vero cells. The median effective doses were 8.6 and 7.8 μM, respectively. The drug was less potent than acyclovir and other related antiviral drugs, but it had a high therapeutic index against both HSV-1 and HSV-2. Drug-resistant viruses were readily produced in cell culture. These variants were cross-resistant to acyclovir, 2′-fluoro-5-iodoaracytosine, and 2′-fluoro-5-methylarauracil but were susceptible to vidarabine or phosphonoformate. These findings confirm that the selective antiviral activity of 5-ethyl-2′-deoxyuridine is mediated by the virus-induced thymidine kinase. Oral or intraperitoneal administration of the drug at nontoxic doses was ineffective in protecting mice against intracerebral challenge with virus. Using implanted osmotic minipumps or coadministering the drug with dimethyl sulfoxide failed to decrease the mortality rate. In guinea pigs infected genitally with HSV-2, topical drug treatment was more effective than placebo in reducing lesion severity and other clinical and virological variables. These effects were noted whether the drug treatment was initiated 3 or 24 h after infection (ascertained serologically). Drug-treated animals had a significantly lower herpes antibody titer than did placebo-treated guinea pigs, suggesting that the drug can also reduce the viral antigen load. In this model, the drug appeared to be as effective as topical phosphonoformate or acyclovir.

5-Ethyl-2′-deoxyuridine (EdU) is a pyrimidine nucleoside with activity in cell culture against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and vaccinia virus (9, 16, 28, 48). The compound is also active in rabbits with herpes keratitis (13, 15), in mice with systemic herpetic infection (7, 8), and in dorsally infected guinea pigs (46). Studies in humans have indicated positive effects with this drug for the treatment of ocular herpes (13, 35). EdU does not appear to interfere with regeneration of the epithelium of the rabbit eye (17). EdU has no demonstrable mutagenicity, and the drug does not induce RNA retroviruses in several cell culture systems (20, 29, 45, 48). In rats, more than 90% of the drug or its metabolites are excreted in the kidney within 24 h (24).

Since the full spectrum of activity of this drug against HSV is incomplete, several studies were initiated which had the following objectives: (i) delineating the activity of EdU against 2 laboratory strains and 12 clinical isolates of HSV-1 and 1 laboratory strain and 12 clinical isolates of HSV-2; (ii) comparing the cell culture activity of EdU with those of acyclovir (ACV), vidarabine (ara-A), *E*-5-(2-bromovinyl)-2′-deoxyuridine, 2′-fluoro-5-methylarauracil (FMAU), and 2′-fluoro-5-iodoaracytosine against laboratory strains of HSV; (iii) assessing activities of several of these agents against EdU-resistant variants; (iv) determining the capacity of EdU administered in various regimens and dosage forms to protect mice after intracerebral challenge with HSV-1 and HSV-2; and (v) comparing the activities of topical EdU, phosphonoformate (PFA), and ACV against HSV-2-induced genital infections in guinea pigs.

(Part of this work was presented at the 13th Annual UCLA Symposium on Herpesvirus, Keystone, Colo., 8 to 13 April 1984; see J. Cell. Biochem. Suppl. 8B, p. 206, abstr. no. 1384, 1984.)

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MATERIALS AND METHODS

Viruses. The F strain of HSV-1 and G strain of HSV-2 were obtained from B. Roizman (University of Chicago). The KOS strain of HSV-1 was obtained from D. Coen (Harvard University). The MS strain of HSV-2, used in the guinea pig experiments, was isolated from a patient with multiple sclerosis by M. Gudnatottir (University of Iceland) and was chosen for these experiments because the virus is less virulent in the guinea pig than other HSV-2 variants and because it is known to produce a disease characterized by repeated reactivations (47). These viruses were cloned and high-titered pools were prepared in HEp-2 cells, as previously described (40). The MS virus was sensitive to EdU in plaque reduction assays (50% effective dose [ED_{50}] = 7.1 μM). The clinical isolates were collected from patients in Emory University-associated hospitals (Atlanta, Ga.) and were grown in secondary rabbit kidney cells. High-titered pools ($\geq 10^8$ PFU/ml) were prepared in HEp-2 cells. All of the clinical isolates were obtained before the licensing of ACV in the United States (pre-1982). The MS and F strains of HSV were used to prepare viral antigen for serological assays (6).

Drug-resistant HSV variants were prepared by single passage of virus in Vero cells at a multiplicity of infection of 10^{-4} in the presence of EdU (200 μM) dissolved in a 1% agarose overlay. Individual plaques were picked, and a high-titered pool was prepared in HEp-2 cells. The different viruses were then tested for increased resistance to EdU and related antiviral drugs. The ED_{50} and ED_{90} values for the viruses were estimated from linear regression analysis of plots of percent plaque reduction versus the logarithm of the micromolar drug concentration. Only the linear portion of the raw datum titration curve was used in the analysis. The thymidine kinase (TK) levels induced by the viruses were determined by the method of Cheng et al. (5).

Cell culture and virus assays. Mycoplasma-free Vero cells,

obtained from Flow Laboratories (McLean, Va.), were used for virus isolation and plaque assays. The methodologies for the plaque reduction and cytotoxicity assays have been previously described (40). No deaminase inhibitor was used in plaque assays.

Drugs. The sources of the drugs for cell culture assays were as follows: EdU, Ortho Pharmaceutical (Canada) Ltd.; PFA, Astra Läkemedel AB, Södertälje, Sweden; ACV, Burroughs Wellcome Co., Research Triangle, N.C.; *E*-5-(2-bromovinyl)-2′-deoxyuridine, E. De Clercq, Rega Institute for Medical Research, Leuven, Belgium; and 2′-fluoro-5-iodoaracytosine and FMAU, J. J. Fox, Memorial Sloan-Kettering Cancer Center, Rye, N.Y. Topical EdU (3%) and its corresponding placebo were provided by Ortho Pharmaceutical (Canada) Ltd. This placebo is a water-based cream formulation which is similar to that used in the gynecological cream Delfen. An aqueous formulation of PFA (3%) and its corresponding placebo were obtained from Astra Läkemedel AB. ACV (5%) ointment in polyethylene glycol was obtained from Burroughs Wellcome.

Mouse encephalitis studies. Random-bred Swiss ICR mice (female, 4 to 5 weeks old) were obtained from Harlan-Sprague Co. (Indianapolis, Ind.). After acclimatization for 2 weeks, the mice were inoculated in the right cerebral hemisphere and divided into groups containing 12 to 15 mice per group. The methods used for the virus inoculations, drug treatments (0.5 ml per mouse per dose), and toxicity studies have been described previously in detail (39, 40). After virus inoculation, all mice were checked twice daily for mortality for 21 days. The osmotic minipumps (model 2001), which deliver 1 μl/h for 7 days, were obtained from Alza Corp. (Palo Alto, Calif.).

Guinea pig studies. (i) Primary infection. Female Hartley strain albino guinea pigs (Elm Hill Breeding Laboratories, Chelmsford, Mass.), weighing about 220 g each, were used for these experiments. The animals were acclimatized for 1 week before the experiment. The lighting in the animal room was kept on 12-h cycles of light and dark.

The vaginal closure membrane was ruptured with a sterile cotton applicator, and the vagina was then swabbed with phosphate-buffered saline (PBS). Thirty minutes later, HSV-2 (strain MS) was inoculated intravaginally with a catheter (22 gauge) attached to a micropipette. The dose of virus selected (about 9.7×10^4 PFU/50 μl) caused about 70% of the guinea pigs to have clinical disease, with less than a 3% mortality rate (by day 21 after virus inoculation). Almost 90% of the surviving animals seroconverted by day 21 (see Table 5). The guinea pigs were randomized, ear tagged, and placed two per cage. The groups of animals that received drug or placebo were coded. The code was broken only at the end of the experiment (day 60). All of the animals were monitored daily and scored (see scoring procedure) by the same person, from day 0 through day 60 after the primary infection. The person scoring the animals did not know which animal had received drug or placebo. Only the data obtained on days 2 to 14 inclusive were used to analyze the severity of the primary infection. The animals were weighed weekly for the duration of the experiment.

(ii) Drug administration. The guinea pigs were treated twice daily (9 a.m. and 5 p.m.) for 7 days for a total of 14 doses. The creams or ointments were applied generously to the genital area and lower vagina and then rubbed in with a finger cot (Ansell Inc., Dothan, Ala.). The finger cots were changed between animals to prevent cross-infection. Uninfected guinea pigs were also treated with the drugs to monitor any adverse skin reaction.

Two separate experiments were performed. For the first experiment, the following five virus-inoculated groups were used: EdU placebo, PFA placebo (10 guinea pigs per group), 3% EdU treated 3 h and 24 h after infection (19 guinea pigs per group), and 3% PFA treated 24 h after infection (10 animals per group). Previous pilot studies had shown that there was no difference in the severity of disease between untreated animals and EdU placebo-treated guinea pigs. For the second experiment, the group receiving 3% EdU 24 h after infection was compared with the group receiving topical 5% ACV 24 h after infection (15 animals per group).

(iii) Recurrence rate. Animals were monitored daily for recurrences [presence of external lesion(s) in the genital area only] from days 14 to 60 after the primary infection. In those animals that did not show evidence of primary disease, the first observed lesion was classified as recurrent if the lesions first occurred at least 15 days after animal inoculation. The severity and duration of the lesion(s) and site of recurrence were recorded. A single recurrent episode was defined as the reappearance of lesion(s) after 2 or more lesion-free days (score, <1.5; see below). An area demonstrating only redness was not considered a recurrence. The animals were exposed to the minimum amount of handling during this observation period.

(iv) Scoring procedure. The lesion-disease scoring procedure of Kern et al. (24) was modified as follows: 0 = no symptoms or apparent evidence of virus infection; 0.5 = redness, swelling (without lesions), or both; 1.5 = single, small (<2 mm) discrete vesicle; 2.0 = single, large (≥2 mm) discrete vesicle; 2.5 = several small, discrete vesicles, vaginal ulcers (or bleeding), or both; 3.0 = several large, discrete vesicles; 3.5 = severe swelling of genital area (with lesions); 4.0 = several small or large vesicles coalescing; 4.5 = loss of sphincter control, hind leg paralysis, or both; 5.0 = external ulcers, maceration, or both. When healing began (drying and crusting), the score was reduced by 1. As the lesion(s) healed further, the score declined as the area of involvement was reduced. For example, the following healing scores were given: 4 = generalized crusting covering most of the external genitalia; 3 = multiple, defined crusted lesions; 2 = few crusted lesions; 1 = loss of crusts and reepithelization.

(v) Viral cultures. The guinea pigs were swabbed before being dosed to determine the presence of infectious virus on the morning of days 2, 6, 10, and 20 after virus challenge with a premoistened, sterile cotton-tipped applicator. The applicator was placed in Hanks minimum essential medium (1 ml) containing 10% inactivated newborn calf serum and antibiotics. Samples were frozen at −70°C within 30 min of swabbing or titrated directly in Vero cells. To decrease drug carry-over in drug-treated animals, swabs were collected at least 12 h after previous treatment. The virus titer was determined by plaque assay in Vero cells (40) and is expressed as PFU per milliliter of media in which the swab was placed. To further reduce the potential problem of drug carry-over after adsorption of the test medium, the cells were washed three times with PBS. Swabs from drug-treated uninfected animals were also collected for testing in vitro for residual antiviral activity.

Antibody studies. Under anesthesia with Metofane (Pitman-Moore Co., Washington Crossing, N.J.), the guinea pigs were bled by heart puncture (21-gauge needle) on day 21 after virus inoculation. All sera were stored at −20°C until assayed to confirm infection. The sera were tested for the presence of anti-HSV antibodies by an enzyme-linked immunosorbent assay.

This assay was performed in 96-well Immulon II plates (Dynatech Laboratories, Inc., Alexandria, Va.). Optimal dilution of the viral antigens for coating the wells and the peroxidase-labeled, affinity-purified antibody to guinea pig immunoglobulin G (H + L) conjugate were determined by block titration. The method for preparing the HSV and cell control antigens has been described previously (6). The plates were coated with the HSV or cell control antigen (100 μl) overnight at 4°C in buffer A (35 mM $NaHCO_3$, 15 mM Na_2CO_3 [pH 9.6], 0.02% NaN_3). The next day, the plates were washed twice with buffer B (PBS [pH 7.2], Tween 20 [0.05%]). The serum (100 μl) from virus-inoculated and uninfected guinea pigs was added at a 1:50 dilution to duplicate wells in buffer B containing 1% bovine serum albumin. The plates were sealed and incubated at 37°C. After 1 h, the plates were washed five times with buffer B with an automatic washer (Titertek Microplate Multiwash, Flow Laboratories, Inc., McLean, Va.). One hundred microliters of peroxidase-conjugated goat anti-guinea pig immunoglobulin G (Kirkegaard and Perry Laboratory, Inc., Gaithersburg, Md.) was then added (diluted 1:1,000). After incubation at 37°C for 45 min, the plates were washed five times, and pH 6.0 substrate solution (100 μl) containing 40 mg of *O*-phenylenediamine–2 HCl (Eastman Kodak Co., Rochester, N.Y.) and 0.05% H_2O_2 in 100 ml of substrate buffer (7.8 g of citric acid and 17.9 g of Na_2HPO_4 per liter of water) was added. After 20 min at room temperature, the reaction was stopped with 4 N H_2SO_4 (50 μl). The absorbance was measured at 492 nm with a Titertek Multiscan (Flow Laboratories, McLean, Va.). The plate reader has been interfaced with an Apple II Plus computer for calculation of the mean and standard deviation of the absorbance for each serum. The 1:50 dilution of serum was considered positive when the difference between the mean of the absorbance with the HSV-infected cell extract antigen and the control antigen was ≥0.20. The antibody titer was estimated by titrating the same standard serum from an infected guinea pig for every experiment. Unknown sera that were ambiguous or negative at 1:50 were retested at a dilution of 1:20. A linear regression curve was used to predict the titer of HSV antibody in the test serum, based on the absorbance of the 1:20 or 1:50 dilution.

Statistical analyses. In the analyses presented for the guinea pig studies, only animals that were serologically positive for HSV (titer ≥1:20) were included. Animals that died spontaneously due to viral infection were also excluded (see Table 5). The results related to the primary disease presented in this paper were calculated from data accumulated on day 2, when some of the animals began to have clinical disease, to day 14 after infection, when they sometimes had their first recurrence [defined as the appearance of lesion(s) after more than 2 successive disease-free days]. For analysis purposes, two sets of comparisons were made. Results for animals treated with EdU 3 or 24 h after virus inoculation were compared with each other and with those for guinea pigs treated with the placebo. Results for animals given PFA 24 h after infection were compared with those for recipients of PFA placebo. Results for animals treated with ACV 24 h after virus inoculation were compared with those for animals receiving EdU 24 h after infection (see Table 5, experiment 2). For most comparisons, a parametric analysis of variance could not be used, as the assumptions of the *F* statistic (specifically, variance homogeneity and normally distributed data) were not met. Therefore, the data were analyzed by using nonparametric procedures. A Kruskal-Wallis one-way analysis of variance available in SPSS-X (statistical package for the social sciences) was used to test for differences among the three EdU groups for the four variables defined below. When warranted, paired comparisons were made as described by Marascuilo and McSweeney (34). A Mann-Whitney U test, also available in SPSS-X, was used to compare the two PFA groups and the group receiving ACV 24 h after infection with the group receiving EdU 24 h after infection. Differences in mortality rate were compared with a Fisher exact test. The survival probabilities from drug-treated mice and control groups were compared by using the Breslow (Savage) test, as previously described (39).

Definition of variables. The total number of days the animal had a lesion score of at least 1.5 from day 2 to 14 was defined as the duration of the disease. A second variable, the overall lesion-disease score, was obtained by taking the sum of the daily lesion-disease scores of 1.5 or greater for each animal. The lesion-disease score of each animal from day 2 to day 14, when graphed, provided a curve reflecting the animal disease state over time. By using the trapezoidal method, the area under the curve was computed for each animal. The number of disease-free days was also determined as the number of days that an animal had a lesion score of less than 1.5.

RESULTS

Antiviral and cytotoxicity assays. EdU was tested to determine its antiviral activity against 3 laboratory strains and 24 low-passage clinical isolates of HSV-1 and HSV-2, using plaque reduction assays in Vero cells. The results (Table 1) indicate that the mean ED_{50}s (± 1 standard deviation) were 8.62 ± 1.17 and 7.79 ± 1.25 μM, respectively. This difference was not statistically significant. Thus, EdU was equally effective in inhibiting HSV-1 and HSV-2 strains within a narrow range of ED_{50} and ED_{90} (5.6 to 11.4 and 9.0 to 18.3 μM, respectively). This drug was less active than other experimental and licensed antiviral agents (with the exception of ara-A), and was nontoxic to rapidly dividing Vero cells up to 1,000 μM. The drug had a high therapeutic index (50% inhibitory dose/ED_{90}) for both HSV-1 and HSV-2 strains (Table 2). EdU and ACV were the least toxic compounds tested.

Resistance studies. From single passages of HSV-1 and HSV-2 in the presence of 200 μM EdU, drug-resistant clones were isolated and high-titered pools were made in the absence of EdU. Table 3 shows the susceptibility results in Vero cells obtained with a typical pair of EdU-resistant HSV-2 variants. In comparison with the parent virus, which was cloned in the absence of drug, G-EdU-C1 and G-EdU-C5 were essentially refractory to EdU, ACV, 2′-fluoro-5-iodoaracytosine, and FMAU treatment but responded to ara-A or PFA. The EdU-resistant clones were found to be TK^-, in that they induced less than 0.5% of the TK activity of the parent clone.

Mouse studies. When groups of 12 mice were inoculated intracerebrally with about four 50% lethal doses of HSV-2 (strain G, equivalent to about 20 PFU), 75% of the PBS-treated group died with a mean day of death (MDD) of 8.0 (Table 4, experiment 1). Treatments with EdU intraperitoneally 5 h after infection at doses of 20 to 800 mg/kg per day resulted in no significant increase in MDD or survival compared with PBS-treated mice. In contrast, administration of ara-A or ACV resulted in a significant increase in the MDD. Dosage with ACV also significantly decreased the mortality rate. Similar results were obtained in mice inoculated intracerebrally with HSV-1 (KOS strain). For example, when groups of 12 mice were treated with PBS, EdU (800

TABLE 1. Sensitivity of laboratory strains and clinical isolates of herpes simplex viruses to EdU in plaque reduction assays in Vero cells

HSV type and isolate no.	Origin	EdU (μM)	
		ED_{50}	ED_{90}
HSV-1[a]			
F	Lab strain	10.0	16.9
KOS	Lab strain	8.0	12.5
81–647	Brain	10.7	16.2
81–1445	Brain	8.4	15.4
81–1038	Mouth	7.5	9.5
0346	Mouth	10.0	16.8
80–555	Mouth	7.5	12.3
81–963	Sputum	6.0	9.0
81–691	Chin	8.1	9.9
80–533	Back	5.6	8.9
80–654	Rectum	11.4	17.3
80–646	Cervix	10.0	16.1
80–424	Vulva	10.1	16.4
80–951	Labia	7.4	9.6
Mean ± SD		8.62[b] ± 1.77	13.34 ± 3.40
HSV-2[a]			
G	Lab strain	8.2	16.7
81–1039	Arm	5.7	11.0
81–1061	Finger	8.9	14.2
81–1214	Back	7.9	15.3
81–1120	Penis	6.8	15.3
81–1170	Penis	6.1	15.1
81–1273	Penis	8.3	15.6
81–1252	Genital	7.8	16.4
672	Genital	7.3	10.7
81–1260	Cervix	10.4	16.0
81–1238	Vulva	8.5	16.5
81–1345	Vulva	8.5	18.3
81–925	Vulva	6.9	10.4
Mean ± SD		7.79[b] ± 1.25	14.73 ± 2.50

[a] Determined by immunofluorescence (5).
[b] T-statistics evaluation (HSV-1 versus HSV-2): $ED_{50} - t = 1.41$; df = 25, $P = 0.17$; $ED_{90} - t = 1.20$; df = 25, $P = 0.24$.

mg/kg), and ACV (60 mg/kg), the mortality was 100, 75, and 42%, respectively (data not shown). Oral EdU treatments had essentially no effect on the mortality rate or on the MDD when mice were infected with either HSV-1 (data not shown) or HSV-2 (Table 4). Attempts to increase the bioavailability of EdU by using a subcutaneously implanted osmotic minipump or by coadministering the drug with dimethyl sulfoxide also failed to increase either survival or the MDD (Table 4, experiment 2).

Intraperitoneal doses of EdU up to 1,600 mg/kg per day were not toxic to uninfected mice. No deaths, decrease in weight, or failure to gain weight was noted in these age- and weight-matched animals (data not shown). Dimethyl sulfoxide (up to 20% [vol/vol]) and EdU (800 mg/kg) in combination with dimethyl sulfoxide were also nontoxic to mice.

Primary disease in guinea pigs. The clinical course of the infection was similar to that described by other investigators (32, 42, 43, 47). Unlike other workers, we established infection "take" by a serological assay. Genital herpetic lesions usually developed by day 4 and progressed in EdU placebo-treated guinea pigs to form ulcers by day 7. The percentage of guinea pigs that exhibited primary lesions and seroconversion was about the same for drug- and placebo-treated animals (Table 5, experiment 1).

The primary infection cleared more rapidly with application of EdU than with placebo (Table 5, experiment 1); the median duration of disease (score >1.5) was reduced from 6.3 days in placebo-treated animals to 2.8 and 2.2 days in groups treated with EdU 3 and 24 h after infection, respectively. Disease duration was significantly different for the three treatments (Kruskal-Wallis; $\chi^2 = 8.5$; $P < 0.02$). Paired comparisons indicate that disease duration was significantly longer for the placebo group than for either of the drug-treated groups ($P < 0.05$). However, no differences were found when the two EdU treatments were compared with each other ($P > 0.05$).

The severity of primary lesions, as measured by the total lesion score (>1.5), was reduced from a median of 15.0 in the EdU placebo group to 4.3 and 2.0 in the groups treated with EdU at 3 and 24 h after infection, respectively (Kruskal-Wallis; $\chi^2 = 9.03$; $P < 0.02$). Paired comparisons indicated that the data for both of the drug-treated groups were significantly different from the EdU placebo group ($P < 0.05$). The lower scores in drug-treated animals reflect fewer and smaller nonprogressive lesions. The data distributions for the two EdU treatment groups were not different ($P > 0.05$).

The median duration of primary disease was also reduced from 9.0 days in placebo groups to 5.1 and 4.8 days in the treated groups. The median number of disease-free days (score <1.5) for the EdU groups (10.3 and 10.8 days) was almost twice as great as that for the EdU placebo group (6.8 days) (Kruskal-Wallis; $\chi^2 = 8.5$, $P < 0.02$). The drug-treated groups were significantly different from placebo groups ($P < 0.05$). Again, no differences existed between the two EdU treatments ($P > 0.05$).

Figure 1 shows the differences in the areas under the mean disease score-day curves for drug-treated and placebo groups. The median area in the EdU placebo group of 16.8 was more than twice the areas in the EdU-treated groups (Table 5), a difference that was significant ($\chi^2 = 9.77$, $P < 0.01$). The areas for the two EdU treatment groups, however, were not different ($P > 0.05$).

For both EdU treatments, the mean duration of lesions and total lesion scores was shortened or reduced by more than 60% when compared with EdU placebo, suggesting that late treatment (24 h) was as effective as early (3 h) treatment.

PFA (3%) cream, used as a positive control for these

TABLE 2. Antiviral activity, cytotoxicity and therapeutic index of EdU relative to other licensed and experimental drugs against HSV in plaque assays in Vero cells

Drug	Antiviral activity (μM) against:				Cytotoxicity (ID_{50} [μM])[a]	Therapeutic index (ID_{50}/ED_{90}) against:	
	HSV-1 (F)		HSV-2 (G)				
	ED_{50}	ED_{90}	ED_{50}	ED_{90}		HSV-1 (F)	HSV-2 (G)
EdU	10.0	16.9	8.2	16.7	1,500	89	90
FMAU	0.028	0.061	0.033	0.11	8	131	73
FIAC[b]	0.023	0.048	0.030	0.084	22	458	262
BVDU[c]	0.019	0.062	0.23	17.0	191	3,081	11
ACV	0.071	0.31	0.043	0.36	1,600	5,161	4,444
Ara-A	30.3	59.8	50.0	94.7	60	1	0.6

[a] ID_{50}, Concentration of antiviral drug that will inhibit 50% of cell growth on day 3 in rapidly dividing Vero cells.
[b] FIAC, 2'-Fluoro-5-iodoaracytosine.
[c] BVDU, *E*-5-(2-Bromovinyl)-2'-deoxyuridine.

TABLE 3. Cross-resistance studies with EdU-resistant HSV-2 variants derived from strain G

Virus	Antiviral activity (ED_{50} [μM]) (fold increased resistance)[a]						
	EdU	ACV	FMAU	FIAC	Ara-A	PFA	TK phenotype[b]
G (clone)[c]	8.2	0.6	0.03	0.03	45.0	30.0	TK^+
G-EdU-C1	44.8 (5)	2.5 (4)	6.3 (210)	2.7 (90)	93.5 (2)	30.5 (1)	TK^-
G-EdU-C5	128.7 (16)	42.9 (72)	0.83 (28)	5.2 (173)	50.6 (1)	28.5 (1)	TK^-

[a] Fold increased resistance = ED_{50} of resistant variant/ED_{50} cloned parent virus.
[b] Determined as described in reference 5.
[b] The cloned virus was prepared in the absence of drug. This virus was more resistant to ACV than the parent virus (see Table 2) but was within the range of ED_{50} for HSV-2 isolates to be classified as ACV susceptible (see reference 41).

experiments, had a significant therapeutic activity when compared with the EdU placebo group (Fig. 1). However, the differences between the group treated with PFA 24 h after infection and its corresponding placebo were not statistically significant for any of the variables measured (Table 5).

In the second experiment comparing the group receiving EdU with the group receiving ACV treatments 24 h after infection, statistical analyses for the variables showed no difference between the two drug-treated groups (Table 5, experiment 2). Despite the large variability observed in this experiment, the data for the EdU treatment group 24 h after infection on the duration of disease, area under the curve, and total lesion score were comparable to those obtained in experiment 1.

Recurrent disease in guinea pigs. The overall rate of recurrences for the 60-day observation time was 44.6% for the 82 animals that seroconverted. The mean duration of the first episode was 2.3 days. No differences in rate or duration of recurrence were noted in the drug-treated animals versus control groups or each other. For example, the rate for the EdU placebo group and the groups receiving EdU 3 and 24 h after infection was 43, 38, and 33%, respectively. Infectious virus could not be isolated from any of the recurrent lesions that were cultured.

Antiviral activity and serology. Topical EdU had an effect on virus shedding from the genital tract of infected guinea pigs when compared with EdU placebo (Table 6). By day 6 after virus inoculation, 83% of EdU placebo animals were still shedding virus compared with 40% of the animals receiving EdU 24 h after infection and 12% of the animals receiving EdU 3 h after infection. There was no significant difference in the peak median virus titer between the different groups of animals (5×10^3 PFU/ml in placebo versus 25 and 15 PFU/ml in the animals receiving EdU 24 and 3 h after infection, respectively) (Mann-Whitney, $P > 0.05$). PFA treatment appeared to be more effective in reducing virus shedding; by day 6, whereas virus could be recovered in some of the EdU-treated animals, none was isolated from any of the PFA-treated guinea pigs (data not shown). There was no evidence for drug carry-over, as determined by plaque reduction assays, in vaginal swabs taken from drug-treated, uninfected guinea pigs.

Of the animals that seroconverted, the median estimated antibody titers in the groups receiving EdU 3 and 24 h after infection were 1:239 and 1:197, respectively, whereas the titer for the EdU placebo group was 1:1,600 (Table 6). These differences were statistically significant. PFA treatment also markedly reduced the HSV antibody titer compared with the PFA placebo group. However, because of the smaller number of animals which seroconverted in these two groups, statistical significance was not reached (Table 6).

Weight and dermal toxicity. The animals receiving drug or placebo gained 8 to 11% additional weight by the end of the treatment period compared with day 0 and continued to gain weight during the subsequent 5 weeks (data not shown). By that time, their average weight had increased 69 to 82% compared with day 0. The differences in weight of the group at weekly intervals after virus inoculation were not statistically significant. In addition, no apparent skin irritation was observed in uninfected guinea pigs treated topically with the placebo, EdU, and PFA creams.

DISCUSSION

The antiherpetic activity of EdU has been confirmed in these studies on 3 laboratory strains and 24 clinical isolates of HSV-1 and HSV-2. EdU was not toxic to rapidly dividing Vero cells at 1 mM. The order of antiviral activity, as determined in this report by plaque reduction assays in Vero

TABLE 4. Effect of intraperitoneal and oral EdU in mice inoculated intracerebrally with HSV-2 (G)

Treatment[a]	Dose (mg/kg per day)	MDD ± 1 SD[b]	Mortality [no. mice dead/no. treated (%)][b]
Expt 1			
PBS (virus control)	0	8.0 ± 3.4[e]	9/12 (75)
EdU (i.p.)	20	7.1 ± 2.5[e]	8/12 (67)[e]
	40	7.3 ± 2.2[e]	12/12 (100)[e]
	60	8.8 ± 2.7[e]	12/12 (100)[e]
	100	7.4 ± 2.2[e]	9/12 (75)[e]
	200	8.4 ± 1.8[e]	10/12 (83)[e]
	400	8.4 ± 2.1[e]	11/12 (92)[e]
	800	9.8 ± 2.1[e]	8/12 (67)[e]
ACV (i.p.)	60	13.5 ± 5.3[d]	4/12 (33)[d]
Ara-A (i.p.)	60	11.6 ± 4.9[d]	5/12 (42)[e]
EdU (oral)	80	7.3 ± 3.0[e]	10/12 (83)[e]
	160	7.9 ± 1.9[e]	10/12 (83)[e]
	480	11.1 ± 2.5[e]	7/11 (64)[e]
	800	8.8 ± 1.9[e]	5/12 (42)[e]
Expt 2			
PBS (virus control)	0	6.1 ± 2.0[e]	12/12 (100)[e]
EdU (minipump)	420	6.0 ± 1.4[e]	7/7 (100)[e]
DMSO (i.p.)	15[c]	5.6 ± 2.9[e]	15/15 (100)[e]
EdU (i.p.)	800	7.6 ± 2.6[e]	13/15 (87)[e]
EdU-DMSO (i.p.)	800-15[c]	7.8 ± 4.4[e]	13/13 (100)[e]

[a] Given 5 h after intracerebral inoculation. Dose scheduling for intraperitoneal (i.p.) treatment, twice a day for 4 days; oral treatment given ad libitum for 4 days. The osmotic minipumps were inserted subcutaneously.
[b] Calculated on day 21.
[c] Percent (vol/vol).
[d] Probability that the observed increase in survivor number or increase in MDD was due to chance, $P < 0.05$.
[e] Not significant.

TABLE 5. Effects of topical EdU and PFA on the severity of HSV-2 infection in the guinea pig genital model

Variable[a]	Expt 1					Expt 2	
	EdU placebo	3% EdU after 3 h	3% EdU after 24 h	PFA placebo	3% PFA after 24 h	3% EdU after 24 h	5% ACV after 24 h
Primary lesion(s) (no. with/total [%])	8/10 (80)	16/19 (84)	18/19 (95)	9/10 (90)	7/10 (70)	9/15 (60)	11/15 (73)
Mortality (no. dead/total)[b]	1/10	1/19	1/19	0/10	1/10	1/15	1/15
No. of animals which seroconverted	7	16	18	9	7	11	14
Mean duration of disease (days) ± 1 SD	8.00 ± 3.82	3.19 ± 2.71	2.89 ± 2.32	5.22 ± 3.87	3.29 ± 3.68	3.64 ± 3.50	3.50 ± 2.88
Median	6.3	2.8[c]	2.2[c]	4.0	1.3[d]	3.0	3.2
Mean total lesion/disease score ± 1 SD	24.00 ± 16.25	8.22 ± 11.26	6.11 ± 5.91	14.17 ± 14.23	8.29 ± 9.91	8.91 ± 6.63	9.93 ± 9.69
Median	15.0	4.3[c]	2.0[c]	7.0	2.5[d]	10.5	5.8
Mean area under the lesion/disease–day score curve ± 1 SD	23.72 ± 14.68	9.56 ± 10.58	8.17 ± 5.90	15.03 ± 13.03	9.43 ± 10.25	10.57 ± 10.54	11.20 ± 9.38
Median	16.8	6.4[c]	5.8[c]	8.3	4.5[d]	8.8	7.3
Mean no. of days disease free ± 1 SD	5.57 ± 3.74	9.81 ± 2.71	10.11 ± 2.32	7.78 ± 3.87	9.71 ± 3.68	9.36 ± 3.50	9.50 ± 2.88
Median	6.8	10.3[c]	10.8[c]	9.0	11.7[d]	10.0	9.8

[a] For definitions of the variables measured, see Materials and Methods.
[b] By day 21 after virus inoculation.
[c] Significantly different from the corresponding placebo by paired comparison using the ranking of the Kruskal-Wallis or Mann-Whitney nonparametric procedure; $P < 0.05$.
[d] Not significant; $P > 0.05$.

cells, agrees well with those of Teh and Sacks (49). However, they found significant differences between HSV-1 and HSV-2 (mean ED_{50} = 2.13 and 1.21 μM, respectively) in a cytopathic effect assay with BHK-21 cells. The range of susceptibility of the HSV-1 and HSV-2 clinical isolates to EdU was remarkably narrow (ED_{50} = 5.6 to 11.4 μM). This finding is unlike that obtained with other antiviral drugs, such as ACV (41), for which differences of 10-fold or more in ED_{50} values among clinical isolates are common.

The mechanism of action of EdU has not been studied in detail. Antiviral properties of EdU are probably mediated by the HSV-encoded TK. After selective phosphorylation of the drug by the viral TK and further phosphorylation by cellular enzymes, EdU-5′-triphosphate may incorporate into virus DNA as counterfeit thymidine, although the evidence for this is not conclusive (19, 26). To confirm the role of the viral TK in activating this drug, EdU-resistant HSV variants were prepared in cell culture. These viruses were essentially refractory to some nucleoside analogs (ACV, 2′-fluoro-5-iodoaracytosine, and FMAU) which require the viral TK for activation and were devoid of this virus-induced enzyme (Table 3). However, the viruses were susceptible to drugs that interact with the viral DNA polymerase (PFA and ara-A). These findings confirm that the selective antiviral activity of EdU is dependent on the presence of the virus-induced TK (4). So far, none of the resistant viruses obtained in the cell culture experiments had an altered substrate specificity (TK^+ and EdU resistant). These results suggest that the predominant phenotype of the resistant viruses that may be isolated clinically will be of the TK-deficient type.

TABLE 6. Mean and median peak vaginal virus titer and HSV antibody titer of guinea pigs infected with HSV-2 and treated with EdU, PFA, or their corresponding placebos

Treatment group	Log_{10} peak virus titer (PFU/ml)		Reciprocal log_{10} HSV antibody titer[a]	
	Mean ± SD	Median	Mean ± SD	Median
EdU placebo	3.75 ± 3.64	3.70	3.21 ± 2.97	3.20
3% EdU after 3 h	3.24 ± 3.54	1.18[c]	2.72 ± 2.81	2.38[b]
3% EdU after 24 h	3.32 ± 3.58	1.40[c]	2.96 ± 3.21	2.29[b]
PFA placebo	3.49 ± 3.62	2.53	3.01 ± 2.90	3.14
3% PFA after 24 h	3.13 ± 3.54	2.11[c]	2.68 ± 2.76	2.12[c]

[a] Animals were bled on day 21 after virus inoculation.
[b] Significantly different from corresponding placebo; $P < 0.05$.
[c] Not significant; $P > 0.05$.

Oral and intraperitoneal treatments with EdU were ineffective in Swiss mice infected intracerebrally with various doses of HSV-1 or HSV-2. The drug was essentially inactive even at daily doses of 800 mg/kg and nontoxic at doses up to 1.6 g/kg. In correlated studies not presented here, high-pressure liquid chromatographic analyses of serum extracts from mice which had received 400 mg of EdU per kg intraperitoneally revealed that after 10 min the majority of the drug was converted to 5-ethyluracil, a metabolite devoid of antiviral activity. Additionally, EdU could not be detected in the brain of mice. It is, therefore, not surprising to find that EdU was essentially inactive in the mouse HSV encephalitis model. The relatively poor in vivo activity of EdU may also be attributed to its rapid degradation to 5-ethyluracil by the ubiquitous thymidine phosphorylase which is present in many tissues of mice and other rodents (22, 27, 52, 55). This rapid catabolism has also been observed in guinea pigs receiving oral EdU (D. Ilse, Ortho Pharmaceutical, personal communication), suggesting that EdU is not likely to be useful for oral treatment of cutaneous herpetic infections.

Two different approaches were taken to increase delivery of EdU to the target organ and to maintain high levels of EdU in the serum of mice. Broadwell et al. (3) had shown that dimethyl sulfoxide can reversibly open the blood-brain

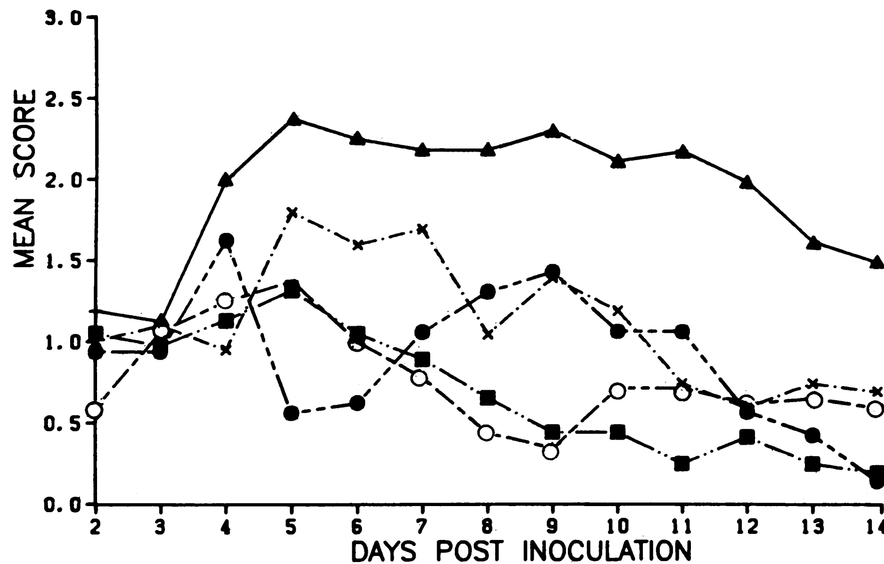

FIG. 1. Mean lesion-disease score in guinea pigs infected with HSV-2 and treated with topical EdU placebo (▲), PFA placebo (×), 3% EdU after 3 h (○), 3% EdU after 24 h (■), and 3% PFA after 24 h (●).

barrier of mice to the enzymatic tracer horseradish peroxidase. This approach to drug delivery to the infected organ could allow a lipid-insoluble drug, such as EdU, to cross the blood-brain barrier. Although this approach gave positive results in pilot studies, these findings could not be reproduced in subsequent experiments with larger groups of mice (Table 4, experiment 2). The second approach took advantage of the high aqueous solubility of EdU (>80 mg/ml) and involved the use of subcutaneously implanted osmotic minipumps to maintain a constant infusion of EdU in the mice. Unfortunately, this approach was not effective (Table 4).

Topical 3% EdU cream was evaluated in a blind fashion in guinea pigs infected genitally with HSV-2. Such infections closely resemble the disease in humans and have provided useful indicators of drug efficacy in the treatment of human genital herpes (1, 10, 12, 14, 23–25, 31–33, 36–38, 42–44, 46, 50, 53). The results indicate that topical application of EdU in guinea pigs caused significant alterations in the development of lesions and severity of disease. EdU was effective in shortening the median duration of disease and rapidity of clearing of primary lesions. There was no significant difference between groups receiving EdU at 3 or 24 h after infections (ascertained serologically). However, early therapy appeared to cause decreased shedding of virus towards the end of therapy, suggesting that this modality may reduce viral load.

EdU appeared to be as effective as topical 3% PFA or 5% ACV, formulations known to be effective in this model (23, 24, 31, 36–38). The difference between the PFA-treated group and PFA placebo was not as great as that obtained with EdU and its placebo or when PFA was compared with EdU placebo. It appears that the PFA placebo may have some therapeutic activity. Since the chemical composition of this placebo is proprietary information, it was not possible to explore the components responsible for this unexpected result. Nevertheless, the results obtained with topical PFA were very similar to those reported by Mayo et al. (36). In that study, however, PFA treatment was initiated 72 h after virus inoculation, and the animals which died during the course of the experiment were included in their analysis.

A novel aspect of this report was to exclude from the analyses guinea pigs that did not seroconvert. Since antiviral drugs may influence the clinical course of the disease and prevent the recovery of virus from the vagina, positive antibody response to HSV is a particularly useful objective parameter for determining infectivity. Although the development of humoral immunity in guinea pigs infected with HSV has been studied (11, 54), the effect of antiviral drug treatments has not been reported previously. Almost 90% of the surviving animals seroconverted by day 21 after virus inoculation (Table 5). A significant decline (about sevenfold) in the median HSV antibody titer was noted in animals receiving EdU 3 or 24 h after infection compared with placebo when measured on day 21 after infection (Table 6). However, by day 60, this difference was not apparent (data not shown), suggesting that the antiviral drug reduced the viral antigen load and thus delayed development of high antibody titers. It is likely that subsequent clinical or subclinical recurrences may boost the HSV antibody response. These results also suggest that EdU therapy may interfere with the immune response to the virus infection in some animals, as had been first reported in HSV-infected mice treated early with effective doses of the antiviral drug FMAU (39). EdU itself has been found to have no immunosuppressive activity (18). The decrease or delay in the restoration of the humoral or cellular response has recently been reported to occur in humans undergoing systemic ACV therapy (2, 30, 51). The clinical implications of these findings are as yet unclear.

The pattern and duration of recurrences were determined in the guinea pigs that seroconverted. There was no statistical or clinical difference in the rate of recurrences in the various treated groups and their corresponding placebos (Fischer exact test). Whereas the duration of the first recurrence ranged from 1 to 3 days, the duration of the second recurrence usually lasted a day; about 45% of the animals in the various treatment groups had one or more recurrences. Consistent with the findings of Scriba (42), virus could not be isolated from scrapings from recurrent lesions or detected by electron microscopy of vesicular fluid. In contrast, Stanberry and co-workers (47) reported that infectious virus

could be isolated from 31% (4 of 13) of recurrent lesions. This discrepancy may be attributed to the differences in virus preparation or source of the Hartley guinea pigs. The short duration of the recurrence and high levels of complement in the lesions may reduce the chance of virus detection.

In conclusion, all of the data in this study suggest that topical 3% EdU is significantly more effective than placebo in treating primary genital herpes in guinea pigs. Its antiviral activity in this model was similar to that produced by ACV and PFA. The apparent lack of toxic effect of this drug, its rapid degradation to 5-ethyluracil in mice and guinea pig sera, and its ability to penetrate the skin (46) make topical EdU a good candidate for clinical trials. Whether such therapeutic effects will also be observed in humans awaits the results of further trials.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Ortho Pharmaceutical (Canada) Ltd., by a Merit Award from the Veterans Administration, and by Public Health Service grant RO1-A1-18600 from the National Institutes of Health.

We thank A. C. Solling-Jorgenson for excellent technical assistance.

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