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# In vitro and in vivo antiviral activity of 2′-fluorinated arabinosides of 5-(2-haloalkyl)uracil

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**Summary**

5-(2-Fluoroethyl)-2′-deoxyuridine (FEDU), its 2′-fluoroarabinofuranosyl analog (FEFAU) and the 2′-fluoroarabinofuranosyl analog (CEFAU) of the potent anti-herpesvirus compound 5-(2-chloroethyl)-2′-deoxyuridine (CEDU) were evaluated for activity against herpes simplex virus type 1 (HSV-1) and HSV-2 in vitro and in vivo. FEDU, FEFAU and CEFAU proved to be potent and selective anti-herpesvirus agents in vitro. Their potency is evident from their low minimum inhibitory concentrations for HSV-1 and HSV-2, and their selectivity is attested by the marginal inhibition of cell proliferation at relatively high concentrations, and by the high concentrations at which DNA-, RNA- or protein synthesis in normal uninfected host cells is inhibited. Their activity spectrum is broader than that of CEDU: in addition to being highly effective against HSV-1 replication, these derivatives, in particular FEFAU, inhibit HSV-2 replication at concentrations comparable to acyclovir (ACV).

In the systemic and cutaneous HSV-1 infection models in mice, FEDU, FEFAU and CEFAU were markedly less potent than CEDU in suppressing the development of lesions and in reducing the mortality rate. In HSV-2 infections in mice and in guinea pigs FEDU, FEFAU and CEFAU were virtually ineffective. CEDU, however, exerted a protective effect in these animal models, albeit at relatively high concentrations.

Herpes simplex virus type 1 (HSV-1); Herpes simplex virus type 2 (HSV-2); 5-(2-Fluoroethyl)-2′-deoxyuridine; 2′-Fluoroarabinosyl pyrimidine analog

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## Introduction

The class of 5-substituted pyrimidine nucleoside analogs has yielded many actual or candidate antiviral drugs (De Clercq, 1984a). Two of the most potent and selective representatives of this class, 5-(2-bromovinyl)-2′-deoxyuridine (BVDU) (De Clercq et al., 1979) and the recently described 5-(2-chloroethyl)-2′-deoxyuridine (CEDU) (Griengl et al., 1985), effectively inhibit herpes simplex virus type 1 virus (HSV-1) and varicella zoster virus (VZV) replication in vitro (De Clercq et al., 1979, 1982; De Clercq and Rosenwirth, 1985; Griengl et al., 1985; Rosenwirth et al., 1985). Herpes simplex type 2 virus (HSV-2) replication is affected at considerably higher concentrations of CEDU or BVDU than is HSV-1. CEDU is effective against systemic HSV-1 infections in several animal models at a 10-fold lower dosage than are BVDU and acyclovir (ACV) (De Clercq and Rosenwirth, 1985; Rosenwirth et al., 1985). Modification of the sugar moiety of 5-substituted pyrimidine nucleoside analogs has also led to compounds possessing antiviral activity: i.e. analogs containing a 2-deoxy-2-fluoro-β-D-arabinofuranosyl moiety instead of 2-deoxyribose (Watanabe et al., 1979, 1983, 1984; Fox et al., 1982; Perlman et al., 1985; Su et al., 1986). These compounds are potent and selective inhibitors of HSV-1, HSV-2, VZV and cytomegalovirus (CMV) replication (Fox et al., 1982; Lopez et al., 1980; Colacino and Lopez, 1983; Mar et al., 1984). The most active congeners of this series are 2′-fluoro-5-iodo-(β-D-arabinofuranosyl)cytosine (FIAC), 2′-fluoro-5-iodo-(β-D-arabinofuranosyl)uracil (FIAU) and 2′-fluoro-5-methyl-(β-D-arabinofuranosyl)uracil (FMAU). They show, however, cytotoxic effects at considerably lower concentrations than BVDU, CEDU or ACV.

The synthesis and biological properties of the 5-fluoro-analog of CEDU, FEDU, and of the 2′-fluoro-arabinofuranosyl analogs of CEDU and FEDU, referred to as CEFAU and FEFAU, have been described (H. Griengl, E. Wanek, W. Schwarz, W. Streicher, B. Rosenwirth and E. De Clercq, J. Med. Chem., in press). Herein we report a more detailed evaluation of the in vitro and in vivo antiviral activities of these new pyrimidine nucleoside analogs. Their antiherpesvirus activity was compared with that of the reference compounds BVDU, ACV, dihydroxypropoxymethylguanine (DHPG), FIAC, FIAU, FMAU, 5-iodo-2′-deoxyuridine (IDU) and 9-(β-D-arabinofuranosyl)adenine (araA). FEDU, CEFAU and FEFAU proved to be effective inhibitors of HSV-1 and HSV-2 in vitro. In vivo, however, they were much less efficacious than CEDU in various HSV-1 infection models, and virtually inactive in HSV-2 infection models. CEDU, surprisingly, proved more effective in treatment of experimental HSV-2 infections in vivo than might have been expected from its in vitro activity.

## Materials and Methods

### *Test compounds*

The structural formulas of the test compounds CEDU, CEFAU, FEDU and FEFAU are presented in Fig. 1. They were synthesized at the Sandoz Forschungs-

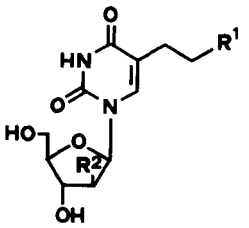


Fig. 1. Structural formulae of CEDU, CEFAU, FEDU and FEFAU.

| $R_1$ | $R_2$ | Compound |
|---|---|---|
| Cl | H | CEDU |
| Cl | F | CEFAU |
| F | H | FEDU |
| F | F | FEFAU |

institut by the method described recently by Griengl et al. (1985; and H. Griengl, E. Wanek, W. Schwarz, W. Streicher, B. Rosenwirth and E. De Clercq, J. Med. Chem., in press). BVDU was synthesized by R. Busson (Rega Institute for Medical Research) and at the Sandoz Forschungsinstitut by the method of Jones et al. (1979). IDU was obtained from Ludeco (Brussels, Belgium), ACV (acycloguanosine; Zovirax) was from Wellcome Research Laboratories (Research Triangle Park, NC, U.S.A.) and was synthesized at the Sandoz Forschungsinstitut by the method of Schaeffer et al. (1978). 9-(1,3-Dihydroxy-2-propoxymethyl)guanosine (DHPG) was synthesized according to Martin et al. (1983). 2′-Fluoro-5-iodo-1-β-D-arabinofuranosylcytosine (FIAC), 2′-fluoro-5-iodo-1-β-D-arabinofuranosyluracil (FIAU) and 2′-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) were obtained from Dr. J.J. Fox, Sloan Kettering Institute for Cancer Research, New York, NY, U.S.A., through the aid of Dr. I. Ghazzouli, Bristol-Myers Company, Syracuse, NY, U.S.A. Adenine-9-β-D-arabinofuranoside (araA) was purchased from Sigma Chem. Comp., St. Louis, MO, U.S.A.

All test compounds were readily soluble, at the highest concentrations used, in cell culture medium for the in vitro experiments and in phosphate-buffered saline (PBS) for the in vivo experiments.

*Viruses*

The origins of the HSV-1 (KOS), HSV-1 (F), HSV-1 (McIntyre), HSV-2 (G), HSV-2 (196), HSV-2 (Lyons), vaccinia virus, and vesicular stomatitis virus strains have been described previously (De Clercq et al., 1980). Virus stocks were prepared in primary rabbit kidney cell cultures, and their titers were determined by plaque formation in Vero cells. HSV-1 (Brand), originally isolated by H. zur Hausen in Erlangen (F.R.G.) and HSV-2 (K979), isolated by B. Vestergaard in Copenhagen (Denmark), were kindly provided by M. Scriba, Vienna (Austria). Stocks of these strains were prepared and their titers determined in Vero cells.

*Mice*

Either Naval Medical Research Institute (NMRI) mice (weight 18–20 g) or hairless (hr/hr) mice (weight 18–20 g) were used for the in vivo experiments. The NMRI mice were randomly bred; the hairless mice were bred by backcross and intercross of the homozygous parents. The NMRI mice were obtained from Charles River Wiga, Sulzfeld (FRG), whereas the hairless mice were from Bomholtgard, Ry (Denmark). The mice were housed under conventional conditions in groups of twelve with unrestricted access to food and water.

*Guinea pigs*

Outbred female 'Pirbright-White' albino guinea pigs (weight = 230–270 g) were used for the in vivo experiments. They were obtained from Savo-Ivanovas Versuchstierzuchtanstalt (Kisslegg, F.R.G.). The guinea pigs were housed under conventional conditions in groups of five and given food and drinking water ad libitum.

*In vitro tests*

The technique for measuring inhibition of HSV-induced cytopathogenicity in vitro (in primary rabbit kidney cells) has been described previously (De Clercq et al., 1980). HSV-1 and HSV-2 plague reduction assays were performed as follows. Vero cell monolayers were infected with serial 10-fold dilutions of the virus stocks. After virus adsorption, cell culture medium containing 0.1% of an HSV-2 antiserum prepared in guinea pigs and various concentrations of the test compounds were added. The number of plaque forming units (PFU) was determined after staining at 46 h postinfection, and was compared with that of the untreated control.

For measuring inhibition of cell growth Vero or HEp-2 cells suspended in culture medium containing test compound at various concentrations were seeded into 24-well tissue culture dishes (Vero: $5 \times 10^4$ cells/well; HEp-2: $1 \times 10^5$ cells/well); four wells were used per variable. After 1, 2, 3, and 4 days aliquots of the cells were stained with 0.01% neutral red in phosphate-buffered saline (PBS) for 2 h at 37°C, washed with PBS, and dried. The stained cells were dissolved in 1.5 ml/well ethanol/0.1 M $NaH_2PO_4$ (1/1), and the optical density of the solution was measured at 546 nm. The absorbance was found to be proportional to the cell number.

The procedure for monitoring the incorporation of radiolabeled DNA, RNA and protein precursors has been described previously (De Clercq et al., 1978). The radiolabeled precursors used were [*methyl*-$^3$H]thymidine ([*methyl*-$^3$H]dThd), [1′,2′-$^3$H]deoxyuridine ([1′,2′-$^3$H]dUrd), [5-$^3$H]uridine and [4,5-$^3$H]leucine. They were all obtained from the Radiochemical Centre (Amersham, U.K.), and their specific radioactivities were 50, 27, 30 and 46 Ci/mmol, respectively.

*In vivo tests*

For systemic treatment of systemic HSV-1 or HSV-2 infection NMRI mice were inoculated intraperitoneally (i.p.) with HSV-1 (Brand) at $1.3 \times 10^5$ PFU/0.1 ml per mouse or with HSV-2 (K979) at $4.7 \times 10^3$ PFU/0.1 ml per mouse and treated either perorally (p.o.) (via gavage) or i.p. twice a day (at 9 a.m. and 4 p.m.) with the indicated doses of the test compound for 5 days, starting on the day of virus infection. Statistical significance of the differences in the final mortality rates was assessed by the $\chi^2$-test with Yates correction for small numbers (Sacks, 1984a). The 50% effective doses in vivo were calculated by the method of Spearman-Kaerber (Sacks et al., 1984b).

The procedure for topical treatment of cutaneous HSV-1 or HSV-2 infection in hairless mice has been recently described (De Clercq, 1984b). The mice were inoculated intracutaneously in the lumbosacral area with either HSV-1 (Brand) at $1 \times 10^6$ PFU/0.025 ml per mouse or HSV-2 (K979) at $1.8 \times 10^5$ PFU/0.025 ml per mouse. The test compounds were formulated in AZDMSO (5% azone [1-dodecylazacycloheptan-2-one], synthesized at the Sandoz Forschungsinstitut by the method of Swain et al. (1953), in dimethyl sulfoxide). They were applied topically four times a day (at 9 a.m., 11 a.m., 2 p.m. and 4 p.m.) for 5 days, starting immediately after virus infection.

For intravaginal treatment of genital HSV-1 or HSV-2 infection, guinea pigs were pretreated on day –5 and –3 with 200 μg per 100 g body weight of β-estradiol 3-benzoate in 0.1 ml sesame oil given subcutaneously. On day 0 they were inoculated intravaginally with HSV-1 (Brand) at $1.3 \times 10^6$ PFU/0.1 ml per guinea pig or with HSV-2 (K979) at $7 \times 10^4$ PFU/0.1 ml per guinea pig. The test compounds were formulated in a mixture consisting of 30–38% (wt/wt) DMSO (depending on the drug concentration), 50% (wt/wt) PEG 400 and 10% (wt/wt) Aërosil (Silicium dioxydatum dispersum) and applied intravaginally two times a day for 5 days starting 6 h after virus infection.

## Results

*In vitro antiviral activity*

In primary rabbit kidney cell cultures FEDU and CEFAU inhibited the replication of HSV-1 at an MIC of 0.25–0.82 μg/ml, that is 2- to 4-fold higher than the MIC of CEDU. FEFAU was about equally active as CEDU against two of the three HSV-1 strains. CEDU, as reported previously (De Clercq and Rosenwirth, 1985; Rosenwirth et al., 1985), proved as potent as IDU and 10-fold less potent than BVDU. Thus, none of the CEDU-related analogs was superior to CEDU against HSV-1 (Table 1).

Against HSV-2 replication, which was inhibited by CEDU only at a concentration about 10-fold higher than that required for inhibition of HSV-1, CEFAU, FEDU and, in particular, FEFAU showed higher activity than CEDU (Table 1).

TABLE 1

Activities of CEDU, CEFAU, FEDU, and FEFAU and the reference compounds IDU, BVDU, and ACV against HSV-1, HSV-2, vaccinia virus, and vesicular stomatitis virus in primary rabbit kidney cell cultures.

| Compound | MIC (μg/ml)[a] for | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HSV-1 (KOS) | HSV-1 (F) | HSV-1 (McIntyre) | HSV-2 (G) | HSV-2 (196) | HSV-2 (Lyons) | Vaccinia virus | Vesicular stomatitis virus |
| CEDU | 0.14 | 0.16 | 0.2 | 2.1 | 2.5 | 0.43 | 30 | >400 |
| CEFAU | 0.30 | 0.58 | 0.30 | 2 | 2.5 | 1.2 | 185 | 300 |
| FEDU | 0.58 | 0.82 | 0.25 | 1.5 | 1.6 | 0.43 | 17 | >400 |
| FEFAU | 0.2 | 1.0 | 0.2 | 0.2 | 2.0 | 0.2 | 20 | >400 |
| IDU | 0.17 | 0.23 | 0.31 | 0.91 | 0.99 | 0.58 | 0.27 | >400 |
| BVDU | 0.017 | 0.02 | 0.02 | 2.3 | 30 | 4.2 | 13 | >400 |
| ACV[b] | 0.06 | 0.04 | 0.05 | 0.04 | 0.02 | 0.06 | 70 | >400 |

[a] Minimum inhibitory concentration required to reduce virus-induced cytopathogenicity by 50%. Mean values for three independent experiments.

[b] Data for ACV were taken from De Clercq et al. (1980).

CEFAU inhibited HSV-2 replication at an MIC of 1.2–2.5 μg/ml, FEDU at an MIC of 0.43–1.6 μg/ml and FEFAU at an MIC of 0.2–2.0 μg/ml. Thus, FEFAU was more potent against HSV-2 than CEDU and BVDU but less potent than ACV.

All CEDU-related analogs can be considered as selective inhibitors of HSV, since they inhibited vaccinia virus replication only at considerably higher concentrations than those required for inhibition of HSV-1 and HSV-2, and, furthermore, they were completely inactive against vesicular stomatitis virus (Table 1).

CEDU and its congeners were also compared, together with the reference compounds, for their effects on virus plaque formation in Vero cell cultures (Fig. 2). Of the three CEDU analogs, FEFAU inhibited most effectively the plaque forming ability of HSV-1 (Brand); it was slightly superior to CEDU, while the other two derivatives were less effective than CEDU. All test compounds were less potent than BVDU, ACV and DHPG. Of the fluoro-arabino group of derivatives, FIAU was superior to FIAC which, in turn, surpassed FMAU in activity. The concentrations of compounds which effected a 10-fold reduction in the number of HSV-1 plaques were, in the order of (decreasing) antiviral potency: FIAU (0.06 μg/ml), FIAC (0.14 μg/ml), DHPG (0.18 μg/ml), BVDU (0.32 μg/ml), ACV (0.36 μg/ml), FMAU (0.62 μg/ml), FEFAU (1.3 μg/ml), CEDU (1.8 μg/ml), FEDU (2.3 μg/ml), CEFAU (6.2 μg/ml).

In their effect on HSV-2 plaque formation the three CEDU analogs were clearly more active than CEDU and BVDU (Fig. 2). FEFAU again was the most potent of the three CEDU analogs, its inhibitory activity being similar to that of ACV, DHPG and FMAU. The activity of FEFAU was surpassed only by FIAC and FIAU. The concentrations of compounds which effect a 10-fold reduction in the number of HSV-2 plaques were, in the order of (decreasing) antiviral potency: FIAU (0.26 μg/ml), FIAC (1.0 μg/ml), DHPG (2.0 μg/ml), FMAU (2.1 μg/ml), FEFAU (3.2 μg/ml), ACV (4.1 μg/ml), FEDU (8.9 μg/ml), CEFAU (17 μg/ml), BVDU (48 μg/ml), CEDU (100 μg/ml).

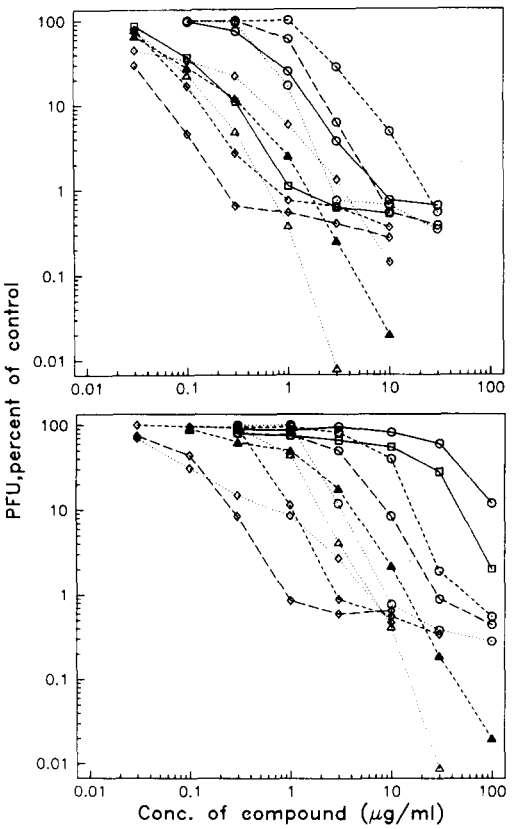


Fig. 2. Antiviral activity of CEDU, CEFAU, FEDU, FEFAU and the reference compounds BVDU, FIAC, FIAU, FMAU, ACV and DHPG against HSV-1 (Brand) (top panel) and HSV-2 (K979) (bottom panel) in plaque reduction assays. The number of PFU was determined in the presence of various concentrations of CEDU (○—○), CEFAU (○---○), FEDU (○– –○), FEFAU (○····○), BVDU (□—□), FIAC (◊---◊), FIAU (◊– –◊), FMAU (◊····◊), ACV (▲---▲), and DHPG (△····△) and is expressed as a percentage of the untreated control.

To estimate the cytotoxic potential of the CEDU analogs in comparison to reference compounds, in particular the fluoro-arabino compounds, growth of Vero and HEp-2 cells in tissue culture was monitored in the presence of various concentrations of the test substances. In general, HEp-2 cells were slightly more sensitive than Vero cells to the growth-inhibitory effects of these nucleoside analogs. At 330 μg/ml, CEDU and its three congeners reduced the cell density at day 4 by 10–40% and thus were less cytotoxic than BVDU and ACV which showed a 50–70% reduction. All other reference compounds were more cytotoxic than BVDU and ACV. Interestingly, DHPG was more inhibitory to Vero cell growth than ACV whereas both acyclic nucleoside analogs were equally cytotoxic to HEp-2 cells.

At 100 μg/ml, CEDU, FEDU, CEFAU and FEFAU and the reference com-

pounds BVDU, ACV and DHPG exerted only minimal inhibitory effects ($\leq$ 35%) on the proliferation of Vero and HEp-2 cells (Fig. 3). At this concentration FMAU and FIAU exhibited 90% inhibition, IDU 65–80%, araA 60–75% and FIAC 60–70%.

At 30 μg/ml, only FIAU and FMAU exerted detectable inhibitory effects on cell growth: FMAU reduced the cell density at day 4 by 70–90% and FIAU by 55–65% (data not shown).

Thus, CEDU and its analogs CEFAU, FEDU and FEFAU are equally, or slightly less, cytotoxic than BVDU, ACV or DHPG, and much less cytotoxic than the fluoroarabinofuranosyl derivatives FIAC, FIAU and FMAU, and the known-to-be less selective nucleoside analogs IDU and araA.

Although inhibitory to HSV-1 and HSV-2 at a concentration of $\leq$ 2.5 μg/ml (Table 1), the three CEDU analogs, CEFAU, FEDU and FEFAU, did not interfere with DNA or RNA synthesis of normal uninfected host cells up to a concen-

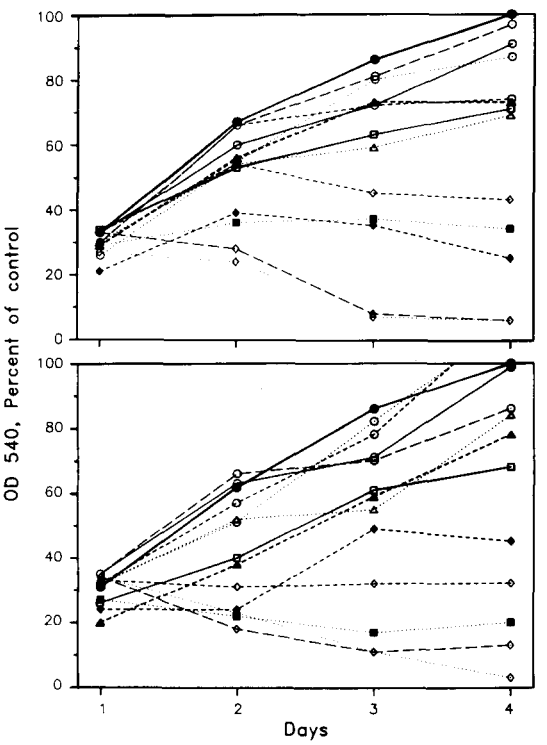


Fig. 3. Growth inhibition of Vero cells (top panel) and HEp-2 cells (bottom panel) by CEDU, CEFAU, FEDU, FEFAU and the reference compounds IDU, BVDU, araA, FIAC, FIAU, FMAU, ACV and DHPG. Cells in suspension were seeded into tissue culture plates at a low density to allow growth for 4 days before becoming contact inhibited. The test compounds were present in the cell culture medium at a concentration of 100 μg/ml. Propagation of cells was monitored by determination, upon staining, of absorbance as described in Materials and Methods. The symbols are as in Fig. 2; in addition IDU (■····■) and araA (♦---♦) are included.

TABLE 2

Effect of CEDU, CEFAU, FEDU, FEFAU, and BVDU on DNA, RNA and protein synthesis in primary rabbit kidney cell cultures.

| Synthesis | Radiolabeled precursor | MIC (μg/ml)[a] | | | | |
|---|---|---|---|---|---|---|
| | | CEDU | CEFAU | FEDU | FEFAU | BVDU |
| DNA | [*methyl*-$^3$H]dThd | 194 (185–205) | 103 (95–110) | 138 (110–170) | 225 (135–305) | 71 (65–76) |
| DNA | [1′,2′-$^3$H]dUrd | 188 (175–208) | 87 (67–105) | 41 (39–44) | 64 (42–86) | 51 (32–76) |
| RNA | [5-$^3$H]uridine | 210 (183–225) | 94 (86–105) | 208 (185–230) | 95 (82–105) | 43 (40–45) |
| Protein | [4,5-$^3$H]leucine | >400 | 158 (130–195) | 360 (300–>400) | >400 | >400 |

[a] Minimum inhibitory concentration required to reduce the incorporation of the radiolabeled precursor by 50%. Mean values for three independent experiments. The range of the individual values is indicated in parentheses.

tration of ⩾ 50 μg/ml (Table 2). CEDU showed antimetabolic activity at approximately 200 μg/ml, CEFAU at approximately 100 μg/ml, FEDU and FEFAU at approximately 50 μg/ml. BVDU impaired normal DNA and RNA synthesis at a concentration of approximately 50 μg/ml. FEDU and FEFAU inhibited the incorporation of dUrd at 4-fold lower concentrations than dThd, which may indicate some inhibitory effect on deoxythymidylate synthase. However, this effect cannot account for the antiviral properties of the compounds, which were observed at much lower concentrations.

The specificity indexes of the three CEDU-analogs, based on the ratio of the MIC for host cell DNA or RNA synthesis (Table 2) to the MIC for HSV-1 replication (Table 1) were 300 (CEFAU), 100 (FEDU) and 250 (FEFAU); for inhibition of HSV-2 replication they were 50 (CEFAU), 50 (FEDU) and 250 (FEFAU). Thus, CEFAU, FEDU and FEFAU can be considered as potent and selective anti-herpes nucleoside analogs.

*In vivo antiviral activity*

CEDU has been shown previously to be highly effective in reducing the mortality rates of mice infected either intraperitoneally, intracerebrally or intracutaneously with HSV-1 (De Clercq and Rosenwirth, 1985; Rosenwirth et al., 1985). To evaluate the in vivo antiviral potentials of CEFAU, FEDU and FEFAU, we compared them with CEDU and the reference compounds BVDU, FIAC, FIAU, FMAU and ACV for their anti-HSV-1 and -HSV-2 activity in systemic (intraperitoneal) and cutaneous experimental HSV infections in mice, and intravaginal infection in guinea pigs.

Peroral administration of CEDU to mice inoculated i.p. with HSV-1 (Brand) resulted in a significant reduction of the mortality rate (Table 3): from 80% to 54% at a dosage of 1 mg/kg per day ($P = 0.017$), 23% at a dosage of 5 mg/kg per day

TABLE 3

Comparative activity of CEDU, CEFAU, FEDU, FEFAU, BVDU, FIAC, FIAU, FMAU and ACV against systemic HSV-1 infection in mice.

| Test compound | Treatment regimen | | Cumulative mortality, percent of infected mice (*P*-value) | Mean survival time in days (*P*-value) | $ED_{50}^{a}$ (mg/kg) |
|---|---|---|---|---|---|
| | Route of administration | Daily dose (mg/kg) | | | |
| CEDU | p.o. | 50, 25 | 0 (< 0.001) | 20.0 (< 0.001) | 1.7 (1.3–2.1) |
| | | 10 | 15 (< 0.001) | 18.7 (< 0.001) | |
| | | 5 | 23 (< 0.001) | 17.9 (< 0.001) | |
| | | 1 | 54 (0.017) | 14.6 (0.005) | |
| CEFAU | p.o. | 100 | 50 (n.s.) | 15.1 (n.s.) | ≈ 100 |
| | | 50 | 50 (n.s.) | 14.1 (n.s.) | |
| | | 25 | 92 (n.s.) | 11.4 (n.s.) | |
| FEDU | p.o. | 50 | 42 (0.026) | 14.8 (n.s.) | 20 (13–31) |
| | | 25 | 58 (n.s.) | 14.0 (n.s.) | |
| | | 10 | 58 (n.s.) | 12.7 (n.s.) | |
| FEFAU | p.o. | 100 | 42 (0.026) | 16.2 (0.016) | 48 (32–71) |
| | | 50 | 50 (n.s.) | 14.6 (n.s.) | |
| | | 25 | 75 (n.s.) | 11.7 (n.s.) | |
| BVDU | p.o. | 100 | 17 (< 0.001) | 17.8 (0.004) | 23 (16–32) |
| | | 50 | 33 (0.006) | 16.8 (0.004) | |
| | | 25 | 54 (n.s.) | 14.2 (n.s.) | |
| | | 10 | 54 (n.s.) | 14.3 (n.s.) | |
| FIAC | p.o. | 25 | 33 (0.014) | 16.8 (n.s.) | 15 (10–21) |
| | | 10 | 58 (n.s.) | 16.2 (n.s.) | |
| | | 5 | 92 (n.s.) | 11.4 (n.s.) | |
| FIAU | p.o. | 25 | 25 (0.002) | 17.9 (0.005) | 10 (7–14) |
| | | 10 | 58 (n.s.) | 13.8 (n.s.) | |
| | | 5 | 58 (n.s.) | 15.3 (n.s.) | |
| FMAU | p.o. | 25, 10 | 0 (< 0.001) | 20.0 (< 0.001) | < 5 |
| | | 5 | 17 (< 0.001) | 18.5 (< 0.001) | |
| ACV | p.o. | 100 | 9 (< 0.001) | 19.4 (< 0.001) | 23 (18–30) |
| | | 50 | 20 (< 0.001) | 17.7 (< 0.001) | |
| | | 25 | 40 (< 0.001) | 16.2 (< 0.001) | |
| | | 10 | 83 (n.s.) | 11.3 (n.s.) | |
| Placebo | p.o. | – | 80 | 11.6 | |
| CEDU | i.p. | 10 | 4 (< 0.001) | 19.6 (< 0.001) | 0.53 (0.43–0.67) |
| | | 5 | 10 (< 0.001) | 19.2 (< 0.001) | |
| | | 1 | 25 (< 0.001) | 17.4 (< 0.001) | |
| | | 0.5 | 29 (< 0.001) | 17.1 (< 0.001) | |
| | | 0.25 | 67 (n.s.) | 14.2 (0.003) | |

| Test compound | Treatment regimen | | Cumulative mortality, percent of infected mice (*P*-value) | Mean survival time in days (*P*-value) | $ED_{50}^{a}$ (mg/kg) |
|---|---|---|---|---|---|
| | Route of administration | Daily dose (mg/kg) | | | |
| CEFAU | i.p. | 100 | 58 (n.s.) | 14.3 (n.s.) | > 100 |
| | | 50 | 67 (n.s.) | 13.1 (n.s.) | |
| FEFAU | i.p. | 100 | 50 (n.s.) | 15.4 (n.s.) | ≈ 100 |
| | | 50 | 67 (n.s.) | 12.7 (n.s.) | |
| ACV | i.p. | 25 | 33 (0.006) | 17.1 (0.006) | < 10 |
| | | 10 | 42 (0.026) | 16.1 (0.008) | |
| Placebo | i.p. | – | 79 | 11.4 | |

[a] $ED_{50}$, 50% effective dose, or dose required to reduce virus induced mortality by 50% (the 95% fiducial limits are indicated in parentheses).

($P < 0.001$), 15% at a dosage of 10 mg/kg per day ($P < 0.001$), and 0% at a dosage of 25 or 50 mg/kg per day ($P < 0.001$). When evaluated under the same conditions CEFAU, FEDU and FEFAU showed considerably less efficacy: the doses required to reduce virus-induced mortality by 50% were 100 mg/kg per day for CEFAU, 20 mg/kg per day for FEDU and 48 mg/kg per day for FEFAU, as compared to 1.7 mg/kg per day for CEDU. Of the reference compounds evaluated under the same conditions, FMAU proved most potent: the mortality rate was reduced from 80% to 17% at a dosage of 5 mg/kg per day ($P < 0.001$) and 0% at a dosage of 10 or 25 mg/kg per day ($P < 0.001$). Thus, its $ED_{50}$ was less than 5 mg/kg per day, which is comparable to the $ED_{50}$ of CEDU and apparently lower than the $ED_{50}$ values obtained for FIAU (10 mg/kg per day), FIAC (15 mg/kg per day) and BVDU or ACV (23 mg/kg per day).

CEDU was even more effective in reducing the mortality rate of mice infected i.p. with HSV-1 (Brand), when it was administered by the i.p. route (Table 3). CEDU brought about a reduction in the mortality rate from 79% to 67% at a dosage of 0.25 mg/kg per day which was not significant; however, the mean survival time was prolonged from 11.4 to 14.2 days at this dosage ($P = 0.003$). At a CEDU dosage of 0.5 mg/kg per day the mortality rate was reduced from 79% to 29% ($P < 0.001$); it was reduced to 25% at a dosage of 1 mg/kg per day ($P < 0.001$), to 10% at a dosage of 5 mg/kg per day ($P < 0.001$) and to 4% at a dosage of 10 mg/kg per day ($P < 0.001$). When evaluated in parallel with CEDU, namely after i.p. administration, neither CEFAU or FEFAU effected a significant reduction in the mortality rate or a significant prolongation of the mean survival time. FEDU proved toxic at a dosage of 50 mg/kg per day, and ineffective at lower doses (data not shown). ACV reduced the mortality to 42% at a dosage of 10 mg/kg per day ($P = 0.026$) and to 33% at a dosage of 25 mg/kg per day ($P = 0.006$) when given i.p.

The low MIC values obtained in vitro against HSV-2 for the 2'-fluoro-arabinosyl derivatives of CEDU and FEDU prompted us to evaluate these compounds in vivo against HSV-2 infection. Neither CEFAU nor FEFAU brought about a significant

reduction in the mortality rate of mice infected i.p. with HSV-2 (K979) when administered p.o. or i.p. (Table 4).

CEDU showed remarkable efficacy against systemic HSV-2 infection: p.o. administration of CEDU resulted in a reduction of the mortality rate from 92% to 74% at a dosage of 100 mg/kg per day which was not significant; however, the mean survival time was prolonged from 9.8 to 14.1 days at this dosage ($P < 0.001$). The mortality rate was reduced from 92% to 49% ($P < 0.001$) at a dosage of 200 mg/kg per day and to 37% at a dosage of 400 mg/kg per day ($P < 0.001$). ACV given p.o. was clearly less effective than CEDU against systemic HSV-2 infection:

TABLE 4

Comparative activity of CEDU, CEFAU, FEFAU and ACV against systemic HSV-2 infection in mice.

| Test compound | Treatment regimen | | Cumulative mortality, percent of infected mice (*P*-value) | Mean survival time in days (*P*-value) | $ED_{50}^{a}$ (mg/kg) |
|---|---|---|---|---|---|
| | Route of administration | Daily dose (mg/kg) | | | |
| CEDU | p.o. | 400 | 37 (< 0.001) | 17.3 (< 0.001) | 142 (110–184) |
| | | 200 | 49 (< 0.001) | 16.2 (< 0.001) | |
| | | 100 | 74 (n.s.) | 14.1 (< 0.001) | |
| CEFAU | p.o. | 80 | 75 (n.s.) | 11.9 (n.s.) | > 80 |
| | | 40 | 67 (n.s.) | 12.9 (n.s.) | |
| FEFAU | p.o. | 100 | 75 (n.s.) | 11.3 (n.s.) | > 100 |
| | | 50 | 92 (n.s.) | 9.6 (n.s.) | |
| ACV | p.o. | 400 | 58 (0.001) | 15.4 (< 0.001) | ≥ 400 |
| | | 200 | 65 (0.015) | 13.9 (< 0.001) | |
| | | 100 | 84 (n.s.) | 11.9 (< 0.001) | |
| Placebo | p.o. | – | 92 | 9.8 | |
| CEDU | i.p. | 400 | 6 (< 0.001) | 19.7 (< 0.001) | 54 (41–72) |
| | | 200 | 44 (< 0.001) | 16.5 (< 0.001) | |
| | | 100 | 47 (0.001) | 16.1 (< 0.001) | |
| | | 50 | 58 (0.018) | 14.9 (< 0.001) | |
| CEFAU | i.p. | 80 | 75 (n.s.) | 12.5 (0.034) | > 80 |
| | | 40 | 100 (n.s.) | 10.2 (n.s.) | |
| FEFAU | i.p. | 100 | 75 (n.s.) | 11.5 (n.s.) | > 100 |
| | | 50 | 100 (n.s.) | 8.4 (n.s.) | |
| ACV | i.p. | 400 | 6 (< 0.001) | 19.5 (< 0.001) | 71 (60–85) |
| | | 200 | 6 (< 0.001) | 19.7 (< 0.001) | |
| | | 100 | 28 (< 0.001) | 17.8 (< 0.001) | |
| | | 50 | 80 (n.s.) | 12.2 (< 0.001) | |
| Placebo | i.p. | – | 84 | 10.8 | |

[a] $ED_{50}$, 50% effective dose, or dose required to reduce virus induced mortality by 50% (the 95% fiducial limits are indicated in parentheses).

it reduced the mortality rate from 92% to 65% at a dosage of 200 mg/kg per day ($P = 0.015$) and to 58% at a dosage of 400 mg/kg per day ($P = 0.001$).

Intraperitoneal treatment of HSV-2 infected mice with CEDU brought about even better protection: at a dosage of 50 mg/kg per day CEDU reduced the mortality rate from 84% to 58% ($P = 0.018$); the mortality rate was reduced to 47% at a dosage of 100 mg/kg per day ($P = 0.001$), to 44% at a dosage of 200 mg/kg per day ($P < 0.001$) and to 6% at a dosage of 400 mg/kg per day ($P < 0.001$). When evaluated in parallel, ACV administered i.p. effected a reduction in the mortality rate from 84% to 28% at a dosage of 100 mg/kg per day ($P < 0.001$) and to 6% at a dosage of 200 or 400 mg/kg per day ($P < 0.001$); thus, ACV was somewhat superior to CEDU when given by the i.p. route.

For topical treatment of cutaneous HSV infection in hairless mice, the test compounds were formulated in AZDMSO, a vehicle that enhances skin penetration (Spruance et al., 1984) and thus is quite appropriate for the topical application of antiviral drugs such as BVDU and ACV (De Clercq, 1984b). ACV is at least as, if not more, effective when applied in AZDMSO than when applied in any other vehicle, such as DMSO (without azone) or the propylene glycol based cream recommended by the manufacturer (De Clercq, 1984b; De Clercq and Rosenwirth, 1985). When applied at 0.3% to the skin of hairless mice infected intracutaneously with HSV-1 (Brand) CEDU significantly suppressed the development of skin lesions (Table 5) ($P = 0.019$), paralysis of hind legs (data not shown), and reduced the mortality rate from 42% to 0% (not significant). The fluoro analog, FEDU, and its 2′-fluoroarabinosyl derivative, FEFAU, both were less potent than CEDU, though at a dosage of 1 and 3%, respectively, a significant reduction in duration of lesions was observed ($P = 0.002$ and 0.001, respectively). FEFAU at a dosage of 3% also reduced mortality from 42% to 0% (not significant). When tested in parallel ACV at a dosage of 1% suppressed the development of lesions ($P = 0.041$) and reduced the mortality rate from 42% to 8% (not significant). Thus, it was less potent than CEDU but surpassed FEDU and FEFAU in activity.

In intracutaneous HSV-2 infection of hairless mice ACV had to be given at a dosage of 3% to effect a significant suppression of lesions and reduction of the mortality rate [from 92% to 17% ($P = 0.001$) for both parameters]. CEDU, surprisingly, was effective at a dosage of 10% in reducing the lesion incidence from 92% to 33% ($P = 0.011$) and the mortality rate from 92% to 8% ($P = 0.001$). The CEDU derivatives which in vitro had shown considerably better anti-HSV-2 activity than CEDU, were essentially inactive in this animal model.

For intravaginal treatment of intravaginal HSV-2 infection of guinea pigs a cream formulation was used which had proved appropriate for this kind of application (M. Schaude, unpublished results). When applied at 5% to the vagina of guinea pigs infected intravaginally with HSV-2 (K979), ACV suppressed lesion development and reduced the mortality rate: only 1 animal out of 10 showed lesions which progressed to paralysis of the hind legs and death. At 2%, ACV effected a marginal protection. FEDU proved toxic also when applied intravaginally: at 5%, 8 out of 10 animals and, at 2%, 5 out of 10 animals died in the first few days of the experiment without any herpetic lesions. Of the surviving animals most developed

TABLE 5

Comparative activity of topically applied CEDU, FEDU, FEFAU and ACV against intracutaneous HSV-1 and HSV-2 infection in mice.

| Virus | Treatment regimen | | Cumulative lesions, percent of infected mice (*P*-value) | Mean duration of lesions in days (*P*-value) | Cumulative mortality, percent of infected mice (*P*-value) | Mean survival time in days (*P*-value) |
|---|---|---|---|---|---|---|
| | Test compound | Dose[a] | | | | |
| HSV-1 | CEDU | 0.3% | 17 (0.019) | 0.9 (< 0.001) | 0 (n.s.) | 20.0 (n.s.) |
| | FEDU | 1% | 42 (n.s.) | 4.5 (0.002) | 25 (n.s.) | 17.3 (n.s.) |
| | FEFAU | 3% | 33 (n.s.) | 3.9 (0.001) | 0 (n.s.) | 20.0 (n.s.) |
| | | 1% | 58 (n.s.) | 6.6 (n.s.) | 25 (n.s.) | 17.3 (n.s.) |
| | | 0.3% | 75 (n.s.) | 6.8 (n.s.) | 33 (n.s.) | 16.8 (n.s.) |
| | ACV | 1% | 25 (0.041) | 2.7 (0.001) | 8 (n.s.) | 19.3 (n.s.) |
| | | 0.3% | 33 (n.s.) | 3.3 (0.002) | 17 (n.s.) | 18.5 (n.s.) |
| | Placebo | – | 75 | 8.3 | 42 (n.s.) | 16.6 (n.s.) |
| HSV-2 | CEDU | 10% | 33 (0.011) | 3.6 (0.002) | 8 (< 0.001) | 19.2 (< 0.001) |
| | FEDU | 1% | 92 (n.s.) | 12.8 (n.s.) | 92 (n.s.) | 10.0 (n.s.) |
| | FEFAU | 3% | 67 (n.s.) | 9.2 (0.009) | 67 (n.s.) | 13.1 (n.s.) |
| | | 1% | 58 (n.s.) | 8.0 (0.006) | 58 (n.s.) | 13.9 (n.s.) |
| | | 0.3% | 58 (n.s.) | 8.0 (0.006) | 58 (n.s.) | 13.8 (n.s.) |
| | ACV | 3% | 17 (0.001) | 2.0 (0.001) | 17 (0.001) | 18.5 (< 0.001) |
| | | 1% | 67 (n.s.) | 7.6 (0.007) | 67 (n.s.) | 14.5 (0.001) |
| | Placebo | – | 92 | 15.7 | 92 | 10.1 |

[a] Expressed in percentage (wt/vol) of active compound in its vehicle (AZDMSO).

lesions and died from the virus infection; thus, no protective effect could be observed with FEDU. CEFAU showed toxic effects only at the higher dosage (5%) and was virtually ineffective. CEDU, in this animal model, again effected a protective effect at a dosage of 10%. No toxic effect was observed at this dosage. Of

TABLE 6

Comparative activity of topically applied CEDU, CEFAU, FEDU, FEFAU and ACV against intravaginal HSV-2 infection in guinea pigs.

| Treatment regimen | | No. of deaths without lesions / no. of guinea pigs infected | No. of guinea pigs with lesions / no. of guinea pigs infected | No. of deaths with lesions / no. of guinea pigs infected |
|---|---|---|---|---|
| Test compound | Dose[a] | | | |
| CEDU | 10% | 0/10 | 6/10 | 2/10 |
| CEFAU | 5% | 2/10 | 8/10 | 5/10 |
| | 2% | 0/10 | 6/10 | 4/10 |
| FEDU | 5% | 8/10 | 2/10 | 2/10 |
| | 2% | 5/10 | 4/10 | 3/10 |
| FEFAU | 5% | 0/10 | 8/10 | 7/10 |
| ACV | 5% | 0/10 | 1/10 | 1/10 |
| | 2% | 0/9 | 5/9 | 5/9 |
| Placebo | – | 0/9 | 9/9 | 6/9 |

[a] The dose is expressed in percentage (wt/wt) of active compound in its vehicle (formulation).

the 10 infected animals, 6 developed lesions and 2 died, as compared to 9 with lesions and 6 deaths in the placebo group.

## Discussion

The present findings indicate that CEFAU, FEDU and FEFAU are potent and selective anti-herpesvirus agents in vitro. Their potency is evident from their low minimum inhibitory concentrations for HSV-1 and HSV-2 (Table 1, Fig. 2), and their selectivity is attested by the marginal inhibition of cell proliferation at relatively high concentrations (Fig. 3), and by the high concentrations at which DNA-, RNA- or protein synthesis in normal uninfected host cells is inhibited (Table 2). The activity spectrum of these derivatives, in particular FEFAU, is broader than that of CEDU: in addition to being highly effective against HSV-1 replication, FEFAU inhibits HSV-2 replication at concentrations comparable to ACV (Table 1, Fig. 2). In a previous study (Perlman et al., 1985) it was found that the 2′-fluoroarabinofuranosyl analog of BVDU (BVFAU) inhibited HSV-2 at a concentration significantly lower than BVDU. Thus, with both bromovinyl and fluoroethyl as the C-5 substituent of the pyrimidine nucleoside, the activity against HSV-2 is increased considerably by introduction of a fluorine in the up position at C′-2 of the sugar ring.

The animal models in which the efficacy of CEFAU, FEDU and FEFAU were evaluated and compared to CEDU and a number of reference compounds (BVDU, ACV, FIAC, FIAU, FMAU) included (1) systemic (i.p.) HSV-1 and HSV-2 infection in NMRI mice, (2) cutaneous HSV-1 and HSV-2 infection in hr/hr mice, and (3) intravaginal HSV-2 infection of guinea pigs. In the systemic and cutaneous HSV-1 infection models in mice, FEDU, CEFAU and FEFAU were markedly less potent than CEDU in suppressing the development of lesions and in reducing the mortality rate. CEDU again proved more effective in vivo than the reference compounds evaluated in parallel (De Clercq and Rosenwirth, 1985; Rosenwirth et al., 1985) except for FMAU, which was about as active as CEDU.

In HSV-2 infections in mice and in guinea pigs, FEDU, CEFAU and FEFAU were virtually ineffective. CEDU, however, exerted a protective effect in these animal models, albeit at relatively high concentrations. When administered perorally it even surpassed ACV in potency, which is known to have a poor oral bioavailability (De Miranda and Blum, 1983).

In the in vivo experiments the test compounds were generally used at doses starting with the maximum tolerated doses. The doses were chosen to allow a direct comparison with the reference compounds. Toxicity was attributed to the compound when it led to death within the treatment period, i.e. before day 6. This occurred with FEDU when administered i.p. at 50 mg/kg/day or intravaginally at 2% or 5% (see Results).

The reasons for the poor anti-herpesvirus activity of FEDU, CEFAU and FEFAU in vivo, especially in HSV-2 infections, are not known. Neither can the excellent in vivo activity of CEDU be rationalized by the in vitro MIC values (De

Clercq and Rosenwirth, 1985; Rosenwirth et al., 1985). Obviously, pharmacokinetic parameters (such as drug absorption, metabolism and elimination; drug levels achieved in blood and tissues) may, to a large extent, determine the antiviral efficacy of this class of compounds in vivo. These pharmacokinetic parameters remain subject of further study.

In conclusion, our present investigations point to the potent and selective antiherpetic activity of a new series of compounds, CEFAU, FEDU and FEFAU. Albeit markedly active against HSV-1 and HSV-2 in vitro, these compounds were not as potent in vivo as could be expected from their in vitro activity. Particularly disappointing was their activity against HSV-2 infections in mice and guinea pigs, which, on the other hand, appeared to respond favorably to relatively high concentrations of CEDU.

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