Susceptibility of Recent Clinical Isolates of Herpes Simplex Virus to 5-Ethyl-2'-Deoxyuridine: Preferential Inhibition of Herpes Simplex Virus Type 2

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We examined the in vitro susceptibilities of three reference strains and 41 recent clinical isolates of herpes simplex virus types 1 and 2 to 5-ethyl-2'-deoxyuridine. This thymidine analog exerts a type 2-preferential but not a type 2-specific antiviral effect. Utilizing a microtiter assay with BHK-21 cells, we found that the mean (± standard deviation) 50% inhibitory dose for herpes simplex virus type 1 isolates was 0.58 ± 0.30 μg/ml as compared with 0.33 ± 0.20 μg/ml for herpes simplex virus type 2 isolates. Isolates were typed according to their susceptibilities to (E)-5-(2-bromovinyl)-2'-deoxyuridine and by an indirect fluorescent-antibody technique in which monoclonal antibody combinations were used. A cytotoxicity assay in which the incorporation of [1',2'-3H]deoxyuridine was measured revealed a 50% inhibitory dose of 37.5 μg/ml, suggesting a favorable therapeutic index for this compound.

Breakthroughs in the field of antiviral chemotherapy during the last few years have largely paralleled the development of thymidine analogs which utilize herpes simplex virus (HSV)-specified thymidine kinase for cellular trapping and activation of these antiviral prodrugs. Most of these compounds have shown either no type specificity or a type preference for HSV type 1 (HSV-1) strains. For example, (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU) has been repeatedly shown to be type 1 specific (5) to the extent that it can now be used as a laboratory tool for typing HSV (10).

Acyclovir (ACV) has demonstrated preference for HSV-1 isolates as well (3), although this finding is variable (5, 17). De Clercq et al. (5), reporting on susceptibilities of a large number of thymidine analogs, suggested that 5-ethyl-2'-deoxyuridine (EdU) may have enhanced activity against HSV-2; however, these observations have not been confirmed with large numbers of clinical isolates. We explored the in vitro susceptibility patterns of 41 recent HSV isolates against EdU. Three reference strains were also examined. We combined two methods of typing clinical strains which we found to be 100% accurate; specifically, BVdU susceptibility (5, 10) and indirect fluorescence with monoclonal antibodies (14).

MATERIALS AND METHODS

**Virus and cells.** HSV isolates were obtained from fresh clinical specimens from patients with oral, geni-
imately 80% cytopathic effect were liberated from the growth surfaces of culture tubes by scraping with a sterile Pasteur pipette. One drop of each cell suspension was placed on a glass slide and allowed to dry. Slides were rinsed in acetone at room temperature for 1 min, followed by fixing in cold acetone at −20°C for 10 min, and dried. Cells were then flooded with 1 drop of a 1:20,000 dilution of monoclonal antibody (HC-1) to HSV-1, and a second cell area was flooded with 1 drop of a 1:20,000 dilution of three monoclonal antibodies (H222, H368, and H379) to HSV-2. Slides were incubated at 37°C for 45 min in a moist environment, washed in three 5-min changes of phosphate-buffered saline (pH 7.6) at room temperature, and then allowed to air dry. Cell areas were again flooded with goat fluorescein-conjugated anti-mouse immunoglobulin (1:100 dilution, Fab fragment; Cappel Laboratorites, Downingtown, Pa.). Slides were then processed as before, mounted with fluorescein-antibody mounting medium (Flow Laboratories), and examined under mercury vapor fluorescence (Nikon, Canada, Inc., Vancouver, British Columbia, Canada).

Antiviral assays. Newly confluent BHK-21 cell monolayers in flat-bottomed microtiter trays (Nunc, Roskilde, Denmark) were washed once with phosphate-buffered saline. Each well was infected with 100 50% tissue culture infectious doses of first-passage virus in 50 μl of medium 199. Virus was adsorbed for 1 h at 37°C. The overlay was then removed, and each well was replaced with 100 μl of drug in serial twofold dilutions ranging from 0.00078 to 25 μg/ml. Trays were then incubated for 48 h at 37°C in the presence of 5% CO2. Assays were stopped when microscopic examination showed 65 to 85% cytopathic effect (48 to 72 h postinfection). The antiviral activity was expressed as the 50% inhibitory dose (ID50), defined as the concentration of antiviral drug required to reduce the viral cytopathic effect to 50% of that of the control microtiter wells, and was calculated by the method of Reed and Muench (16). Each assay was repeated at least eight times.

Cytotoxicity assay. Uninfected BHK-21 cell cultures in microtiter trays were overlaid in quadruplicate with serial dilutions of EdU or ACV in medium 199 and incubated for 48 h at 37°C. Sixteen hours before completion, 0.25 μCi of [1',2',3'H]deoxyuridine (specific activity, 42 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per well was added and incubated at 37°C overnight. Cells were harvested on an eight-chambered cell harvester (Caltech Scientific, Richmond, British Columbia, Canada), collected on filter paper, and dried. The ID50 was defined as the amount of drug required to reduce tritium incorporation by 50%.

RESULTS

All but one of the HSV isolates tested were found to be susceptible to EdU. The mean (± standard deviation [SD]) ID50 for all isolates was 0.46 ± 0.29 μg/ml. A single HSV-1 isolate (strain 229) displayed moderate resistance to EdU, having an ID50 of 1.37 μg/ml, a value more than 2 standard deviations away from the mean.

ID50s of EdU for HSV-1 isolates ranged from 0.16 to 1.37 μg/ml, with a mean ± SD of 0.58 ± 0.30 μg/ml. ID50s of EdU for HSV-2 isolates were somewhat lower, ranging from 0.08 to 0.70 μg/ml, with a mean ± SD of 0.33 ± 0.20 μg/ml (Fig. 1). This enhanced susceptibility of HSV-2 strains was statistically significant (P < 0.01, Student’s t test). By this method, the mean ± SD ID50s of ACV were 0.15 ± 0.06 and 0.24 ± 0.11 μg/ml for HSV-1 and HSV-2, respectively.

Strain differentiation according to monoclonal labeling was compared with BVdU susceptibility. No ambiguity was found in any of the isolates by the indirect fluorescent-antibody technique. All HSV-1 isolates were labeled by HC-1 and not by the H222-H368-H379 mixture. These strains were found to be susceptible to BVdU, with ID50s ranging from 0.001 to 0.006 μg/ml (mean ± SD, 0.003 ± 0.002 μg/ml). HSV-2 strains consistently fluoresced with the H222-H368-H379 monoclonal antibody mixture but failed to fluoresce with HC-1 monoclonal antibody. These isolates were approximately 1,000-fold less susceptible to BVdU, having ID50s ranging from 0.42 to 3.51 μg/ml (mean ± SD, 1.07 ± 0.72 μg/ml).

FIG. 1. ID50s of EdU for HSV-1 and HSV-2 isolates. The mean ID50 of eight assays for each isolate is plotted as the concentration of EdU required to inhibit 50% of control cytopathic effect. A total of 22 strains each of HSV-1 and HSV-2 were examined. The means ± SDs for all isolates of each type are shown to the right of the individual values for each dose used.
Cytotoxicity was assessed by two methods. Microscopic scores were assigned on the basis of cytopathic effect induced by addition of drug. After 48 h of incubation, morphological changes (usually rounding up of cells) were noticeable in some cultures; however, differences were subtle within the midrange, requiring six serial twofold dilutions to effect a change from (mean ± SD) 48.8 ± 4.4 to 64.4 ± 3.2% cytotoxicity (25 to 1,000 µg/ml). The mean (± SD) microscopic cytotoxicity ID₅₀ for EdU was 31.8 ± 14.1 µg/ml.

Cytotoxicity was also measured by comparing the uptake of [1',2'-3H]deoxyuridine. Control cells containing no EdU took up 58.5% of the counts, as did cells exposed to low drug concentrations. Tritium uptake was reduced by 50% (ID₅₀) when cells were exposed to a mean (± SD) concentration of 37.5 ± 3.2 µg/ml. Similar assays with ACV showed a mean (± SD) cytotoxic ID₅₀ of 58.8 ± 16.9 µg/ml.

The therapeutic index for EdU (cytotoxicity ID₅₀/antiviral ID₅₀ ratio) may then be calculated as 65 for HSV-1, 114 for HSV-2, and 81 for all the strains tested. Under these conditions, the therapeutic index for ACV is 392 for HSV-1, 245 for HSV-2, and 309 for all strains tested.

**DISCUSSION**

We report here on the enhanced antiviral efficacy of EdU against HSV-2 as compared with HSV-1 clinical isolates. Previous reports of type specificity for BVDU against HSV-1 (5, 10) were confirmed by our microtiter assay. Despite the clinical demand for enhanced HSV-2 activity, most drugs have demonstrated either equivalent activity against all virus types or enhanced activity against HSV-1. ACV, which has now gained acceptance in the therapy of certain types of genital herpes infections (8), also shows enhanced anti-HSV-1 activity in our assay (ID₅₀, 0.15 µg/ml for HSV-1 versus 0.24 µg/ml for HSV-2). Although there may be a slight variability in susceptibility when it is tested in different cell lines (4), EdU is comparable to ACV as used in our system. In fact, clinical experience in Europe (7, 9) has also suggested some efficacy for this compound.

Interestingly, our resistant isolate was found de novo, without the clinical influence of previous antiviral therapy. This patient had been treated with cortisone cream but had not used topical or systemic antiviral agents at any time. This is consistent with the recent findings of Parris and Harrington (13), who found de novo resistance to ACV in clinical isolates.

EdU depends upon specific utilization by HSV-specified thymidine kinase since it lacks effect against thymidine kinase HSV mutants (5). Furthermore, it is preferentially phosphorylated by HSV thymidine kinase (2). It is incorporated into the DNA of both infected and uninfected cells (15). Further details concerning the mechanisms of action of this agent have not been explored.

Specifically, the mechanism for the enhanced anti-HSV-2 activity of EdU remains unknown. In contrast, the HSV-1-specific activity of BVDU is partially understood. BVDU not only requires the HSV-specified thymidine kinase but may also require an HSV-1-specified thymidylate kinase activity (6), which seems to be unique to HSV-1-infected cells (18), before exerting inhibitory effects at the DNA polymerase level (1).

Cytotoxicity assays such as those described in this study show a wide in vitro therapeutic index for EdU. Cytotoxicity as measured by loss of [1',2'-3H]deoxyuridine incorporation appears to be a sensitive and reproducible marker of cellular cytotoxicity. In contrast, microscopically observable alterations in cellular morphology, as they are induced by drugs, seems to be a crude marker at best. We found a broad concentration range (25 to 1,000 µg/ml) to have morphological effects which were nearly indistinguishable. Although this morphological test has been a useful adjunct in validating radioactivity assays, it probably should be discarded in future studies because it is relatively insensitive and cumbersome. Other methods for assessing cellular integrity, e.g., uptake of vital dyes, have been previously reported (11) and may be helpful adjuncts to radioactivity uptake assays in the future. Furthermore, since vital stains allow for colorimetric analyses, full automation of antiviral susceptibility may be achievable.

EdU is a safe and effective antiviral agent when tested in vitro against recent clinical isolates of HSV. Its effect against HSV-2 is statistically significantly greater than its effect against HSV-1, although there is a considerable amount of overlap.

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**LITERATURE CITED**


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PREFERENTIAL INHIBITION OF HSV-2 BY EdU

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