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Selective Inhibition of the Replication of Herpes Simplex Virus by 5-Halogenated Analogues of Deoxycytidine

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SUMMARY

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5-Bromodeoxycytidine and 5-iododeoxycytidine inhibited the replication of herpes simplex virus as effectively as did 5-bromodeoxyuridine and 5-iododeoxyuridine. However, the 5-halogenated analogues of deoxycytidine were 10–100 times less toxic to uninfected cells than the 5-halogenated analogues of deoxyuridine. The selective action of the halogenated analogues of deoxycytidine appears to be the result of a virus-induced pyrimidine nucleoside kinase, which converts the halogenated analogues of deoxycytidine to halogenated analogues of deoxycytidylate. These results indicate that the 5-halogenated analogues of deoxycytidine are more selective inhibitors of herpes simplex virus replication than the 5-halogenated analogues of deoxyuridine.

INTRODUCTION

A pyrimidine nucleoside kinase induced in herpes simplex virus-infected cells has been shown to catalyze the phosphorylation of 5-bromodeoxycytidine to 5-bromodeoxycytidylate (1). In contrast, the nucleoside kinases of uninfected mouse, hamster, and human cells do not efficiently catalyze the phosphorylation of either 5-bromodeoxycytidine or 5-iododeoxycytidine (1–6).

The pathways of BrdC[4] metabolism in HSV-infected and uninfected cells are illustrated in Fig. 1. In HSV-infected cells BrdC is phosphorylated to BrdCMP, which can then be deaminated to BrdUMP (Fig. 1, pathway B) (1). However, in uninfected

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[4]The abbreviations used are: HSV, herpes simplex virus; BrdC, 5-bromodeoxycytidine; IdC, 5-iododeoxycytidine; BrdCMP, 5-bromodeoxycytidylate; BrdU, 5-bromodeoxyuridine; IdU, 5-iododeoxyuridine; BrdUMP, 5-bromodeoxyuridylate; PFU, plaque-forming units.

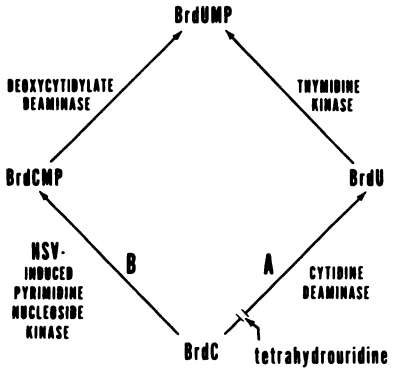

FIG. 1. *Pathways of BrdC metabolism in HSV-infected and uninfected cells*

cells, BrdC is metabolized by deamination to BrdU, which can then be phosphorylated to BrdUMP (Fig. 1, pathway A) (1–6). The conversion of BrdC to deoxyribonucleotides in uninfected cells is prevented by inhibition of cytidine deaminase with tetrahydrouridine (1, 2).

The observation that BrdC is phosphorylated in HSV-infected cells suggested that BrdC and IdC may be more selective inhibitors of HSV replication than BrdU and IdU (1). In this paper we compare the inhibitory effects of 5-halogenated deoxycytidine and deoxyuridine analogues on the replication of HSV and on the replication of uninfected cells.

MATERIALS AND METHODS

Cells. Baby hamster kidney cells, BHK 21/c13 (BHK), and human laryngeal epidermoid carcinoma cells (HEp-2) were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.25 μg/ml of amphotericin B (Fungizone), and either 50 μg/ml of gentamycin or 100 units/ml of penicillin plus 100 μg/ml of streptomycin (growth medium). Cells were grown at 37° in a humidified 5% CO_2 atmosphere.

Viruses. HSV type 1 (HSV-1), strain F, and HSV type 2 (HSV-2), strain G, were purchased from the American Type Culture Collection. These viruses had been grown in HEp-2 cells in the laboratory of Dr. B. Roizman, University of Chicago. Stocks of HSV-2 were grown by infecting BHK cells at a multiplicity of 0.1 PFU/cell and incubating them at 34° for 2–3 days (7). Intracellular virus was harvested by three cycles of freezing and thawing. Virus was assayed by a modification of the method of Roizman and Roane (8). The virus inoculum, 0.25 ml, was added to BHK cells in 60-mm dishes. After 2 hr at 37°, the inoculum was removed and 4 ml of minimum essential medium containing 1% fetal calf serum, antibiotics, and 0.16% pooled human γ-globulin were added. After incubation at 37° for 36–48 hr the medium was removed and plaques were counted after staining with hematoxylin.

Chemicals. 5-Halogenated deoxyribonucleoside analogues were purchased from Sigma Chemical Company. Tetrahydrouridine was kindly supplied by Harry B. Wood, Drug Development Branch, National Cancer Institute.

Inhibition of HSV replication by 5-halogenated deoxyribonucleoside analogues. Virus inoculum, 0.25 ml, was added to cells in 60-mm dishes. After 2 hr the

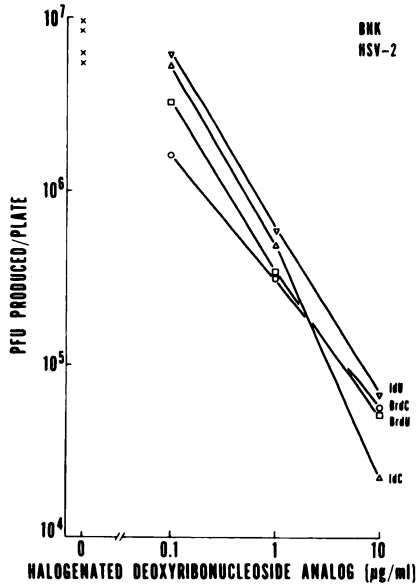

FIG. 2. *Effect of 5-halogenated deoxyribonucleoside analogues on HSV-2 replication in BHK cells*

BHK cells were infected with HSV-2 strain G (0.05 PFU/cell) and incubated for 24 hr in growth medium which contained 5-halogenated deoxyribonucleoside analogues (×, no addition; □, BrdU; ○, BrdC; ▽, IdU; △, IdC). The virus was harvested, and the virus yield was determined by plaque assay.

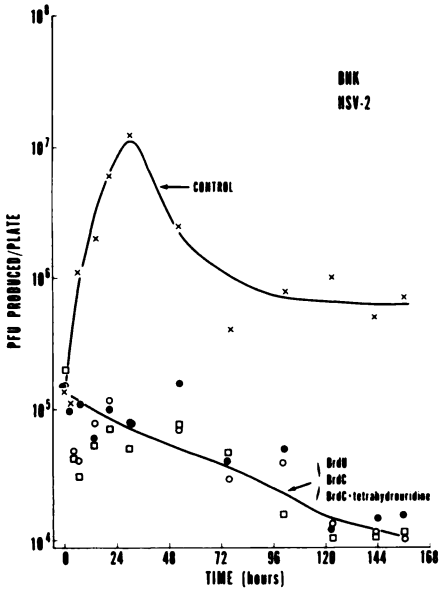

FIG. 3. *Kinetics of HSV-2 replication in BHK cells*
BHK cells were infected with HSV-2 strain G (2.0 PFU/cell). Two hours after infection, the inoculum was removed and the cells were washed twice with phosphate-buffered NaCl solution. Growth medium was added which contained 5-halogenated deoxyribonucleoside analogues at a concentration of 10 μg/ml (×, no addition; □, BrdU; ○, BrdC; ●, BrdC and tetrahydrouridine at a concentration of 100 μg/ml). At the indicated times the virus was harvested, and the virus yield was determined by plaque assay.

inoculum was removed, and 4 ml of growth medium which contained 5-halogenated deoxyribonucleoside analogues were added. After 24–48 hr at 37°, the virus was harvested by three cycles of freezing and thawing of the cells and growth medium in the original 60-mm dish. The virus yield was determined by plaque assay.

Toxicity of 5-halogenated deoxyribonucleoside analogues to uninfected cells. Cells were plated in growth medium at a density of 0.5–1.0 × 10^6 cells/60-mm dish. Twenty-four hours later the medium was removed, and 4 ml of fresh growth medium which contained the indicated 5-halogenated deoxyribonucleoside analogues were added. After 24–48 hr of incubation at 37°, the cells were washed three times with phosphate-buffered NaCl solution and dispersed by treatment with 0.25% trypsin (BHK cells) or 0.05% trypsin–0.02% EDTA (HEp-2 cells). Appropriate cell dilutions were then replated in 60-mm dishes to determine the colony-forming ability of the cells. After 7–9 days of incubation, cell colonies were stained with hematoxylin and counted. The average number of cells per colony was approximately 100; the minimum number of cells defined as a colony was 50. The plating efficiency of untreated cells was approximately 80% for BHK cells and 40% for HEp-2 cells.

RESULTS

Inhibition of HSV replication by 5-halogenated deoxyribonucleoside analogues. We compared the effectivcness of BrdC, IdC, BrdU, and IdU as inhibitors of HSV replication in two cells lines, which differ in their ability to catalyze the deamination of the 5-halogenated deoxycytidine analogues. BHK cells do not contain detectable cytidine deaminase activity (1), so that in HSV-infected BHK cells the conversion of BrdC and IdC to deoxyribonucleotides occurs by their phosphorylation to BrdCMP and IdCMP (Fig. 1, pathway

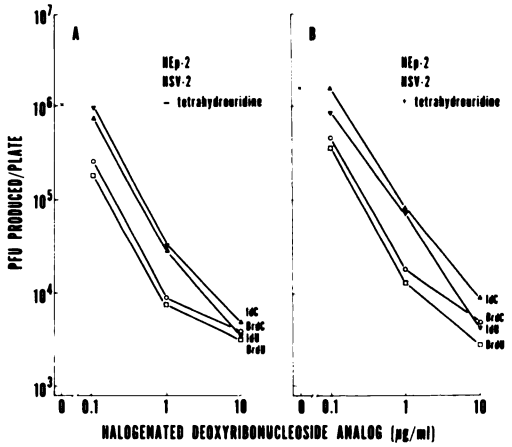

FIG. 4. *Effect of 5-halogenated deoxyribonucleoside analogues on HSV-2 replication in HEp-2 cells*
HEp-2 cells were infected with HSV-2 strain G (0.05 PFU/cell) and incubated for 48 hr in growth medium which contained 5-halogenated deoxyribonucleoside analogues (×, no addition; □, BrdU; ○, BrdC; ▽, IdU; △, IdC) and either no tetrahydrouridine (A) or 100 μg/ml of tetrahydrouridine (B). The virus was harvested and the virus yield was determined by plaque assay.

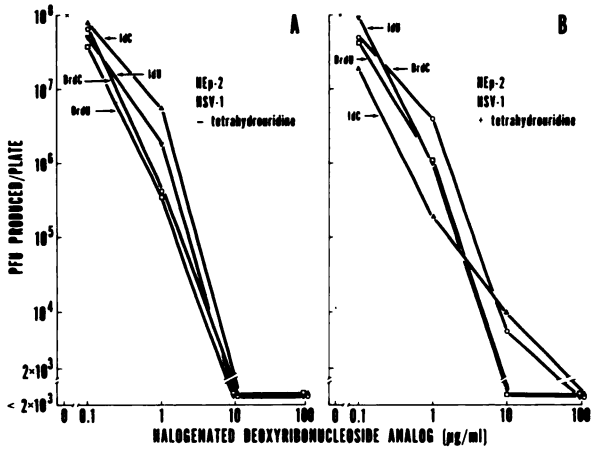

FIG. 5. *Effect of 5-halogenated deoxyribonucleoside analogues on HSV-1 replication in HEp-2 cells*

HEp-2 cells were infected with HSV-1 strain F (0.05 PFU/cell) and incubated for 48 hr in growth medium which contained 5-halogenated deoxyribonucleoside analogues (×, no addition; □, BrdU; ○, BrdC; ▽, IdU; △, IdC) and either no tetrahydrouridine (A) or 100 μg/ml of tetrahydrouridine (B). The virus was harvested and the virus yield was determined by plaque assay.

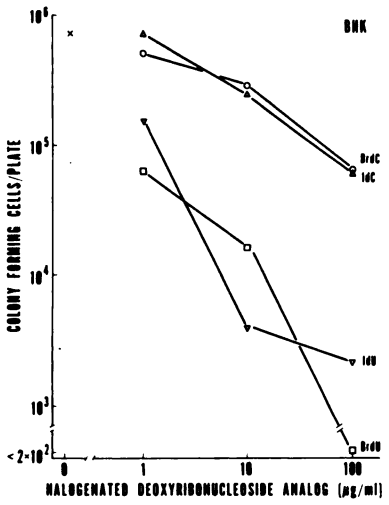

FIG. 6. *Toxicity of 5-halogenated deoxyribonucleoside analogues to uninfected BHK cells*

BHK cells were plated at a density of 5 × 10^5 cells/60-mm dish. Twenty-four hours after plating, the growth medium was removed and replaced with growth medium which contained 5-halogenated deoxyribonucleoside analogues (×, no addition; □, BrdU; ○, BrdC; ▽, IdU; △, IdC). After 48 hr of incubation, the cells were washed three times with phosphate-buffered NaCl and dispersed with trypsin, and appropriate cell dilutions were replated in growth medium. After 7 days of incubation the medium was removed, and the colonies were counted after staining with hematoxylin.

B). HEp-2 cells, however, contain high levels of cytidine deaminase (1). Therefore in HSV-infected HEp-2 cells BrdC and IdC can be converted to deoxyribonucleotides either by phosphorylation to BrdCMP and IdCMP (Fig. 1, pathway B) or by deamination to BrdU and IdU followed by phosphorylation to BrdUMP and IdUMP (Fig. 1, pathway A). In the presence of tetrahydrouridine, BrdC and IdC can be converted to deoxyribonucleotides in either BHK or HEp-2 cells only by phosphorylation to BrdCMP and IdCMP (Fig. 1, pathway B).

The replication of HSV-2 in BHK cells was inhibited to similar extents by BrdC, IdC, BrdU, and IdU (Fig. 2). Significant inhibition of HSV-2 replication was obtained with all four analogues at a concentration of 1.0 μg/ml, and treatment with 10 μg/ml of all four analogues inhibited HSV-2 replication by approximately 99%.

The kinetics of HSV-2 replication in BHK cells is illustrated in Fig. 3. In untreated cells the maximum titer of HSV-2 was obtained approximately 30 hr after infection. In the presence of BrdC or BrdU, no HSV-2 replication was detected for up to 6 days after infection. BrdC was also an effective inhibitor of HSV-2 replication in BHK cells in the presence of tetrahydrouridine (Fig. 3), which was

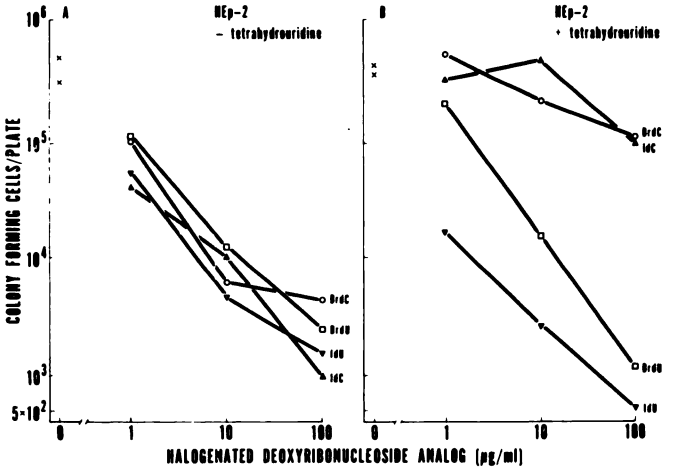

FIG. 7. *Toxicity of 5-halogenated deoxyribonucleoside analogues to uninfected HEp-2 cells*

HEp-2 cells were plated at a density of 1×10^6 cells/60-mm dish. Twenty-four hours after plating, the growth medium was removed and replaced with growth medium which contained 5-halogenated deoxyribonucleoside analogues (×, no addition; □, BrdU; ○, BrdC; ▽, IdU; △, IdC) and either no tetrahydrouridine (A) or 100 μg/ml of tetrahydrouridine (B). After 24 hr of incubation, the cells were washed three times with phosphate-buffered NaCl and dispersed with trypsin, and appropriate cell dilutions were replated in growth medium. After 9 days of incubation the medium was removed, and the colonies were counted after staining with hematoxylin.

added to exclude the possibility that BrdC was deaminated by low levels of cytidine deaminase in BHK cells which were undetectable in previous experiments (1).

Figure 4 illustrates the effect of the 5-halogenated deoxyribonucleoside analogues on HSV-2 replication in HEp-2 cells, which contain high levels of cytidine deaminase. The replication of HSV-2 was inhibited to similar extents by BrdC, IdC, BrdU, and IdU in both the presence and absence of tetrahydrouridine. Similar results were obtained for the effect of the 5-halogenated deoxyribonucleoside analogues on HSV-1 replication (Fig. 5).

These results indicate that BrdC and IdC inhibit the replication of HSV types 1 and 2 as effectively as do BrdU and IdU. The 5-halogenated deoxycytidine analogues are equally effective inhibitors of HSV replication whether they are metabolized by deamination to the 5-halogenated deoxyuridine analogues (Fig. 1, pathway A), as in HEp-2 cells in the absence of tetrahydrouridine, or by phosphorylation to BrdCMP and IdCMP (Fig. 1, pathway B), as in BHK cells and HEp-2 cells in the presence of tetrahydrouridine.

Toxicity of 5-halogenated deoxyribonucleoside analogues to uninfected cells. BrdC and IdC were significantly less toxic to uninfected BHK cells than BrdU and IdU (Fig. 6). Treatment with 1.0 μg/ml of BrdU or IdU reduced the colony-forming ability of uninfected BHK cells by 90%, and treatment with 100 μg/ml of BrdU or IdU reduced the colony-forming ability of BHK cells by more than 99%. In contrast, treatment with 1.0 μg/ml of BrdC or IdC did not significantly alter the colony-forming ability of uninfected BHK cells, and treatment with 100 μg/ml of BrdC or IdC reduced the colony-forming ability of uninfected BHK cells by only 90%.

The results of treating uninfected HEp-2 cells with 5-halogenated deoxyribonucleoside analogues are presented in Fig. 7. Tetrahydrouridine alone had no effect on colony-forming ability. In the absence of tetrahydrouridine BrdC and IdC were as toxic as BrdU and IdU to uninfected HEp-2 cells (Fig. 7A). Since HEp-2 cells contain high levels of cytidine deaminase (1), the toxicity of BrdC and IdC is presumably a result of their deamination to BrdU and IdU (Fig. 1, pathway A). When

the activity of cytidine deaminase was inhibited by tetrahydrouridine, BrdC and IdC were markedly less toxic than BrdU and IdU (Fig. 7B).

DISCUSSION

The results of this study indicate that BrdC and IdC are more selective inhibitors of HSV replication than BrdU and IdU. BrdC and IdC inhibit HSV replication as effectively as BrdU and IdU, but BrdC and IdC are significantly less toxic than BrdU and IdU to uninfected cells.

The activity of BrdC and IdC as inhibitors of HSV replication is consistent with the results of earlier workers (9–12). The antiviral selectivity of BrdC and IdC may be accounted for by the substrate specificity of the HSV-induced pyrimidine nucleoside kinase (1). In HSV-infected cells BrdC and IdC are phosphorylated to BrdCMP and IdCMP (1), whereas these analogues are not phosphorylated in uninfected cells.

To demonstrate the antiviral selectivity of BrdC and IdC, it is necessary to prevent their deamination to BrdU and IdU. In the present study this was accomplished either by using BHK cells, which do not have detectable cytidine deaminase activity (1), or by inhibiting cytidine deaminase with tetrahydrouridine. Since tetrahydrouridine is an effective inhibitor of cytidine deaminase *in vivo* (2, 13), the selectivity of BrdC and IdC as inhibitors of HSV replication may have potential application in the therapy of HSV infections.

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