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Cell Culture Studies on the Antiviral Activity of Ether Derivatives of 5-Hydroxymethyldeoxyuridine

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The antiviral activity of several ether derivatives of 5-hydroxymethyldeoxyuridine against the herpesvirus of infectious bovine rhinotracheitis was determined in monolayer cultures of secondary bovine fetal kidney cells. 5-Methoxy-methyldeoxyuridine (OCH_3UdR) was found to be markedly inhibitory against this virus. Pretreatment of the cells with OCH_3UdR, simultaneous addition of OCH_3UdR with virus to the cells, and postinfection treatment with OCH_3UdR were found to be effective in inhibiting virus-induced cytopathogenic effect. Against this virus, OCH_3UdR was found to be as potent as 5-iododeoxyuridine and cytosine arabinoside. The α-anomer of OCH_3UdR did not show antiviral activity. Preliminary toxicity studies indicate that OCH_3UdR has a very low acute toxicity.

Several pyrimidine nucleosides have been shown to possess inhibitory activity against deoxyribonucleic acid (DNA) viruses (6, 17, 19). 5-Iododeoxyuridine (IUdR) has been shown to be effective in the topical treatment of keratitis due to herpes simplex (9, 11) and vaccinia viruses (12). Other thymidine antimetabolites such as cytosine arabinoside (ara-C) (2), 5-trifluoromethyl-2'-deoxyuridine (7, 10), and 5-ethyl-2'-deoxyuridine (5) have also been reported to possess anti-herpes activity. The therapeutic value of nucleoside antimetabolites in the treatment of viral diseases has been diminished because of their lack of selective action (3, 5, 18).

Bacteriophages have been shown to possess certain unique pyrimidines in their nucleic acids. 5-Hydroxymethyl-2'-deoxyuridylate and 5-hydroxymethyl-2'-deoxycyticylate have been shown to replace thymidylate in the DNA of bacteriophages lytic for *Bacillus subtilis* (8, 16) and *Escherichia coli* (4), respectively. Analogues of these unique pyrimidines (5-hydroxymethyldeoxyribonucleosides) would be particularly useful compounds if they were preferentially incorporated into viral DNA and thus were selectively virotoxic. In this communication, we report the antiviral properties of several ether derivatives of 5-hydroxymethyldeoxyuridine (OHUdR).

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MATERIALS AND METHODS

Cell culture materials, media, and drugs were obtained as follows: disposable plastic microplates and lids (Micro-Test II), Bioquest, Oxnard, Calif.; adhesive-backed clear acetate individual plate sealers and microliter pipettes, Cooke Engineering Co., Alexandria, Va.; infectious bovine rhinotracheitis (IBR) and infectious canine hepatitis viruses, Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada; ara-C, The Upjohn Co., Kalamazoo, Mich.; and IUdR, Sigma Chemical Co., St. Louis, Mo. Ether derivatives were synthesized (1). Commercial growth medium (CulturStat, minimal essential medium MEM, Earle base) containing 10% inactivated fetal calf serum was obtained from Bioquest. Maintenance medium contained the following components by volume: 10% minimal essential Eagle medium (MEM), 10% $NaHCO_3$ (4.4% stock solution), 4% inactivated fetal calf serum, 1% nonessential amino acids, 1% penicillin (100 U/ml), streptomycin (100 μg/ml), and deionized water. Cell cultures used were secondary bovine fetal kidney (BFK) cells and a dog kidney cell line (38th passage) established by Connaught Laboratories, Toronto, Canada. The cells were treated with trypsin or ethylenediaminetetraacetic acid (20), and monolayers of BFK or DK cells were used for all viral chemotherapy experiments.

RESULTS

The design of a primary antiviral chemotherapy experiment is shown in Fig. 1 and is a modification of the method of Sidwell and Huffman (21). Each cup was seeded with 1.6×10^4 cells suspended in 200 μliters of growth medium and incubated in an atmosphere of 5%

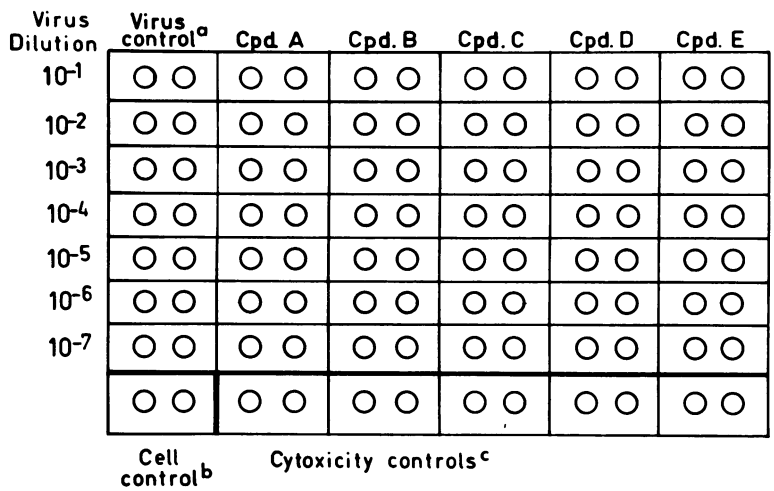

FIG. 1. *Typical layout of a primary chemotherapy experiment using disposable micro tissue culture plates. (a) Cells in maintenance medium with virus only; (b) cells in maintenance medium only; (c) cells in maintenance medium with compounds only. Cpd, Compound being tested for antiviral activity.*

CO_2 at 37 C. When cell monolayers were confluent, 24 to 36 h after seeding, this medium was aseptically poured off and the plates were wiped dry with sterile gauze. During preinfection and simultaneous infection experiments, all compounds were added to maintenance medium as solids at a concentration of 5.04 mg per 18 ml (280 μg/ml; ≈ 10^{-3} M). Subsequently, a 100-fold dilution in maintenance medium was made to obtain a concentration of 2.8 μg/ml (≈ 10^{-5} M). After this, 175 μliters of medium with the appropriate drug concentration was added to each cup either 18 h before or at the same time as virus infection. For postinfection studies, 150 μliters of medium (without nucleoside) was initially added to each cup. Eighteen hours postinfection, 25 μliters of compound prepared in maintenance medium (1,960 and 19.6 μg/ml) was added to give the desired final concentration. These concentrations were selected because ara-C and IUdR exhibited marked antiviral activity at these levels. Serial $\log_{10}$ dilutions of each stock virus (titer, 10^6) were prepared in maintenance medium, and 25 μliters of the appropriate dilution of virus was added to the cups in each experiment. The plates were sealed with adhesive-backed clear acetate sealers, incubated at 37 C for 3 days, and then examined microscopically for cytopathogenic effect (CPE). CPE and cytotoxicity were graded on a scale of 0 (normal cells) to 4 (complete degeneration of the monolayer). The antiviral activity of each compound was determined by inhibition of CPE.

The cytotoxicity of each compound was assessed microscopically by comparing the toxicity controls (compound plus medium) with the cell controls (medium only) and the virus controls (virus plus medium). The drug control cells were scored as follows: increased granularity (1+), slight vacuolation (2+), large holes in the monolayer (3+), and destruction of the cell layer (4+). Any increase in these parameters in the toxicity controls was taken into account in determining the true antiviral effect (degree of inhibition of CPE) of each compound.

The relative antiviral potency of ether derivatives of OHUdR (structural formulas are shown in Fig. 2), IUdR, and ara-C was determined by using secondary BFK cells against IBR virus, and the results of three identical experiments are summarized in Table 1. The methoxy derivative (5-methoxymethyldeoxyuridine [OCH_3UdR]) showed significant antiviral activity at the higher concentration. Butyloxymethyldeoxyuridine (OC_4H_9UdR) exhibited some antiviral activity at the higher concentration. Other ether analogues of OHUdR (OC_2H_5UdR, OC_3H_7UdR, and $OCH_2C_6H_5$) as well as the α-anomer of OCH_3UdR possessed limited or no antiviral activity against IBR virus. OCH_3UdR was found to be as potent as IUdR against IBR virus at both concentrations, and at the lower concentration it was more effective than ara-C. At the higher concentration, ara-C was slightly more potent than the deoxyuridine compounds; however, the antiviral activity was usually accompanied by some cytotoxicity. OHUdR was too toxic (complete disintegration of cells at 2.8 μg/ml), and it was not possible to determine its antiviral activity. Ether derivatives did not produce any observable cytotoxic effects at

concentrations up to 560 μg/ml. At a concentration of 280 μg/ml, IUdR exhibited some activity (one log inhibition of CPE) against ICH virus, but OCH_3UdR failed to show anti-ICH activity.

The influence of time of OCH_3UdR and IUdR treatment on infectivity of virus for the cell monolayer was then investigated by addition of these compounds immediately and 18 h after virus infection. When added simultaneously with virus infection, OCH_3UdR and IUdR showed the same degree of antiviral activity as when added 18 h before virus infection (see Table 1). Similarly, at the higher concentration no difference in antiviral activity was observed when these drugs were added 18 h post-infection. However, at a concentration of 2.8 μg/ml, OCH_3UdR was devoid of activity, whereas IUdR showed slight activity (0.5 log inhibition of CPE). In combination with IUdR or ara-C, OCH_3UdR failed to show synergistic or additive activity when added 18 h before virus infection. In combination experiments, each compound was used at a final concentration of 280 μg/ml.

Preliminary acute toxicity studies using Swiss mice (20 to 25 g) were carried out by dissolving, OCH_3UdR in phosphate buffer (0.15 M, pH 7.2) and intraperitoneal administration of dosages up to 1,000 mg/kg. No mortality was observed in any of the treated groups 1 week post-administration. At post-mortem, no gross lesions were observed.

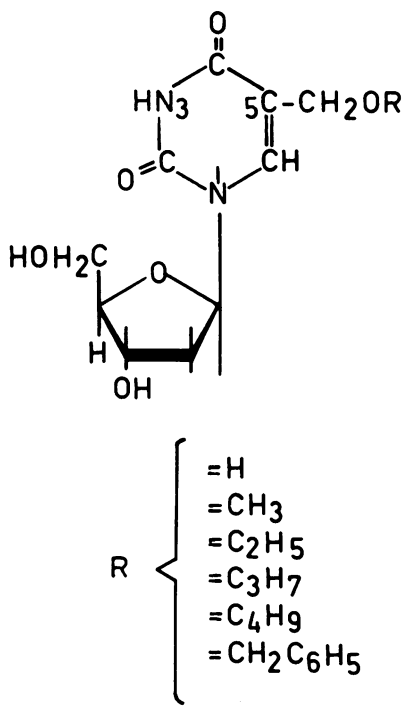

FIG. 2. *Structure of ether derivatives of 5-hydroxymethyldeoxyuridine (R = H).*

TABLE 1. *Relative in vitro antiviral activity of ether derivatives of 5-hydroxymethyldeoxyuridine,[a] IUdR, and ara-C against IBR virus[b] in secondary BFK cells*

Compound[c]	IBR virus titer reductions after exposure to compound at:[d]	
	2.8 μg/ml	280 μg/ml
None	0.0	0.0
5-Hydroxymethyldeoxyuridine[d]	4+ Toxic	4+ Toxic
5-Methoxymethyldeoxyuridine	1.0	2.0
5-Methoxymethyldeoxyuridine (α-anomer)	0.0	0.0
5-Ethoxymethyldeoxyuridine	0.0	0.5
5-Propyloxymethyldeoxyuridine	0.0	0.0
5-Butyloxymethyldeoxyuridine	0.0	1.0
5-Benzyloxymethyldeoxyuridine	0.0	0.5
Thymidine	0.0	0.0
5-Iododeoxyuridine	1.0	2.0
Cytosine arabinoside	0.5	2.5

[a] 1-(2-Deoxy-β-D-ribofuranosyl)-5-hydroxymethyluracil.
[b] 100 tissue culture infective doses per milliliter.
[c] Compounds were added 18 h before virus infection.
[d] Toxic to cell cultures; therefore, antiviral activity could not be determined.

DISCUSSION

The cell culture experiments described here indicate that OCH_3UdR has significant antiviral activity and appears to be as effective as IUdR and ara-C against IBR virus. The range of concentrations of drugs used and the degree of antiviral activity obtained in this system are in general agreement with the results of Miller et al. (13), who used 9-β-D-arabionfuranosyladenine and Buthala (2), who used IUdR and ara-C. The enhanced antiviral activity of ara-C observed at the higher concentration may be due partially to its mild toxicity (1^+ to 2^+) toward BFK cells. Since OCH_3UdR is capable of inhibiting viral CPE when added to cell cultures before, simultaneously with, or up to 18 h after infection in a manner essentially similar to IUdR (6, 15, 18), ara-C (14), and 5-ethyldeoxyuridine (22), inhibition by OCH_3UdR may also occur at a late stage in virus replication. The structure activity data indicate that the presence of bulky alkyl groups at the 5-position of the pyrimidine ring interferes with the incorporation of these compounds into viral DNA. One possible explanation is that higher homologues do not serve as substrates for kinases involved in the biosynthesis of viral DNA. The ability of OCH_3UdR to inhibit viral growth after the cell cultures were infected suggests that it has

potential as a therapeutic antiviral agent. Antiviral activity of OCH_3UdR against other DNA viruses using different cell lines, its mechanism of action, and other biological studies are now in progress.

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