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Effects of *E*-5-(2-Bromovinyl)-2′-Deoxyuridine and Other Selective Anti-Herpes Compounds on the Induction of Retrovirus Particles in Mouse BALB/3T3 Cells

ERIK DE CLERCQ, HUBERTINE HEREMANS, JOHAN DESCAMPS, GABRIEL VERHELST, MARC DE LEY, AND ALFONS BILLIAU

Department of Human Biology, Division of Microbiology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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SUMMARY

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Of a large series of nucleoside analogues, all of which have proven to be effective inhibitors of herpes simplex virus replication in either cell cultures or animals (or both), only a few compounds, viz., 5-propynyloxy-2′-deoxyuridine and 5-iodo- or 5-bromo-2′-deoxyuridine and -2′-deoxycytidine were found to stimulate the release of retro ("reverse transcriptase" or RNA-directed DNA polymerase-containing)-virus particles from mouse BALB/3T3 cells. Many other nucleoside analogues, including the highly selective antiherpes agents acycloguanosine, thymine arabinoside, and *E*-5-(2-bromovinyl)-2′-deoxyuridine, failed to do so. Although the latter observations add further credence to the safety of acycloguanosine, thymine arabinoside, and *E*-5-(2-bromovinyl)-2′-deoxyuridine as antiherpes drugs, they also suggest that these compounds either are not incorporated into BALB/3T3 cell DNA or are incorporated to an extent that does not lead to the expression of oncornaviral genes. This possibility was directly assessed with a radiolabeled analogue of *E*-5-(2-bromovinyl)-2′-deoxyuridine, namely *E*-5-[2-^{125}I-*vinyl*]-2′-deoxyuridine. Under conditions where [5-^{125}I]2′-deoxyuridine was effectively incorporated into BALB/3T3 cell DNA, no such incorporation could be evidenced for *E*-5-[2-^{125}I-*vinyl*]-2′-deoxyuridine.

INTRODUCTION

In recent years various new antiherpes compounds have been developed, some of which show great promise for the treatment of herpes simplex (and varicella zoster) virus infections. This new generation of selective antiherpes agents include compounds such as phosphonoacetate and phosphonoformate (1); acycloguanosine [9-(2-hydroxyethoxymethyl)guanine] (2); thymine arabinoside (araT, 1-β-D-arabinofuranosylthymine) (3); 5-ethyluracil arabinoside (1-β-D-arabinofuranosyl-5-ethyluracil) (4); the 5-substituted 2′-deoxyuridines (dUrd) 5-ethyl- and 5-propyl-dUrd[1] (5, 6); 5-propynyloxy-dUrd (7); 5-methylthiomethyl-dUrd (8); 5-methylsulfonylmethyl-dUrd (9); *E*-5-(2-chlorovinyl)-dUrd, *E*-5-(2-bromovinyl)-dUrd, and *E*-5-(2-iodovinyl)-dUrd (10, 11); the 5-substituted 2′-deoxycytidines (dCyd) 5-bromo- and 5-iododCyd (12); and 5′-amino-5-iodo-2′,5′-dideoxyuridine (5′-amino-5-iodo-2′,5′-ddUrd) (13). Of these nucleoside analogues, *E*-5-(2-chlorovinyl)-, *E*-5-(2-bromovinyl)- and *E*-5-(2-iodovinyl)-2′-deoxyuridine displayed the greatest selectivity as antiherpes agents when assayed in primary rabbit kidneys cell cultures: they inhibited the replication of herpes simplex virus Type 1 (HSV-1) at a concentration that was approximately 10,000-fold lower than the dosage required to affect normal cell metabolism (10, 11, 14, 15).

Most of the aforementioned compounds have proven to be effective in animal model infections, i.e., phosphonoacetate and phosphonoformate in the topical treatment of cutaneous herpes infections in guinea pigs; acycloguanosine, *E*-5-(2-bromovinyl)-dUrd, and 5′-amino-5-iodo-2′,5′-ddUrd in the topical treatment of herpetic keratitis in rabbits; and acycloguanosine, thymine arabinoside, and 5-ethyl-dUrd in the systemic treatment of herpetic encephalitis in mice. Two of these compounds,

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[1] The abbreviations used are: dUrd, 2′-deoxyuridine; dCyd, 2′-deoxycytidine; ddUrd, 2′,5′-dideoxyuridine; dTMP, 2′-deoxythymidine-5′-monophosphate; dTTP, 2′-deoxythymidine-5′-triphosphate; *E* in *E*-5-(2-bromovinyl)-dUrd, *E*-5-(2-iodovinyl)-dUrd, and *E*-5-(2-chlorovinyl)-dUrd stands for *Entgegen* and refers to the position of the halogen in the 2-halogenovinyl side chain.

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acycloguanosine and *E*-5-(2-bromovinyl)-dUrd, have been applied successfully in humans (albeit in uncontrolled clinical trials) in the topical treatment of herpetic keratitis and the systemic treatment of disseminated herpes zoster infections.

Compared with its predecessor 5-iodo-dUrd (idoxuridine), *E*-5-(2-bromovinyl)-dUrd offers the advantage of being both more active and significantly less toxic (10). 5-Iodo-dUrd is indeed a fairly toxic substance; it is teratogenic, mutagenic, and, possibly, carcinogenic. These and other phenotypic effects appear to be correlated with the incorporation of the drug into DNA (16). One of the most notorious effects exerted by 5-halogenated 2′-deoxyuridines is the induction of the release of oncogenic (C-type) viruses from cell lines that normally do not release such viruses (16). In some instances (17), the C-type virus induced by 5-iodo-dUrd was found to be ecotropic, that is, capable of infecting homologous cells, and the infected cells produced carcinomas when inoculated into syngeneic mice.

Few nucleoside analogues have ever been tested for their ability to induce the production of endogenous C-type viruses. This group includes, besides 5-iodo-dUrd and 5-bromo-dUrd, two other 5-substituted 2′-deoxyuridines, 5-fluoro-dUrd (18) and 5-ethyl-dUrd (19), as well as 5-iodo-dCyd and cytosine arabinoside (18). With the advent of several new nucleoside analogues as potentially useful antiviral drugs, i.e., acycloguanosine, thymine arabinoside, and *E*-5-(2-bromovinyl)-dUrd, it seemed of interest to determine whether these nucleoside analogues might also stimulate the release of oncornavirus particles. These investigations may bear not only on the molecular mode of action of these compounds but also on their safety for chemotherapeutic use.

MATERIALS AND METHODS

Cells. BALB/3T3 mouse cells were obtained from S. A. Aaronson (National Cancer Institute, Bethesda, Md.). These seemingly normal murine fibroblasts do not spontaneously produce C-type virus particles. The cells were grown in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.), 2 mM glutamine, and 2 g/liter of $NaHCO_3$ (growth medium).

Test compounds. The origin of the test compounds was as follows: phosphonoacetate, Abbott Laboratories, North Chicago, Ill.; phosphonoformate, Astra Läkemedel AB, Research and Development Laboratories, Södertälje, Sweden, courtesy of E. Helgstrand; acycloguanosine, Wellcome Research Laboratories, Burroughs Wellcome Company, Research Triangle Park, N. C., courtesy of G. B. Elion; adenine arabinoside, Parke Davis and Company, Ann Arbor, Mich., courtesy of R. Wolf (Parke Davis Clinical Research Western Europe, München, West Germany); uracil arabinoside, Sefochem Fine Chemicals, Emek Hayarden, Israel; thymine arabinoside, Terra-Marine Bioresearch, La Jolla, Calif.; 5-ethyluracil arabinoside, see ref. 4; 5-trifluoromethyl-dUrd, Sigma Chemical Company, St. Louis, Mo.; 5-ethyl-dUrd and 5-propyl-dUrd, see ref. 6; 5-propynyloxy-dUrd, see ref. 7; 5-vinyl-dUrd, see ref. 10; 5-ethynyl-dUrd, see ref. 10; E-5-(2-chlorovinyl)-dUrd, *E*-5-(2-bromovinyl)-dUrd, and *E*-5-(2-iodovinyl)-dUrd, see ref. 20; 5-methylthiomethyl-dUrd and 5-methylsulfonylmethyl-dUrd, see ref. 9; 5-bromo-dCyd, Sigma Chemical Company, 5-iodo-dCyd, Serva Feinbiochemica, Heidelberg, West Germany; 5′-amino-5-iodo-2′,5′-ddUrd, Calbiochem, San Diego, Calif.; and 5-iodo-dUrd, Ludeco, Brussels, Belgium.

Radiolabeled compounds. [5-^{125}I]dUrd (specific radioactivity: 1.79×10^3 Ci/mmole or 5 Ci/mg) was obtained from the Radiochemical Centre, Amersham, England. *E*-5-[2-^{125}I-*vinyl*]dUrd was prepared by the direct interaction of *E*-5-(2-carboxyvinyl)-dUrd (20) with ^{125}I. ^{125}I-Labeled NaI served as a source of ^{125}I, from which ^{125}I was generated by the addition of ICl. Therefore, 10 μl of a solution containing 5 mCi/2 nmoles of ^{125}I-labeled NaI were mixed with 50 μl of a solution containing 10 μmoles of ICl, and after 30 min of stirring, a 10-mg molecular sieve (0.4 nm) was added. After stirring for another 30 min, 6 mg (20 μmoles) of *E*-5-(2-carboxyvinyl)-dUrd, 3 mg (30 μmoles) of KOAc, and 40 μl of dimethylformamide were added, and the mixture was further stirred for 20 hr. The reaction mixture was then chromatographed on a 20 × 20 cm SiO_2 plate (solvent, CH_3Cl-EtOH, 80:20), and *E*-5-[2-^{125}I-*vinyl*]dUrd (R_F 0.35) was eluted with 20 ml of ethanol, dried, and dissolved in water. The final product (2 mg) had a specific radioactivity of 1 mCi/mg or 0.38 Ci/mmole.

Induction of retrovirus particles. The cells were processed according to the procedure of Wu *et al.* (21). BALB/3T3 cells were seeded in 60-mm Falcon plastic petri dishes at a density of approximately 10^5 cells/Petri dish, grown for 24 hr, and then incubated with growth medium containing a given concentration of the test compound (5 ml/Petri dish) for another 24 hr. The cell culture medium was then collected (Harvest 1), the cells were washed three times with Eagle's minimal essential medium and further incubated with fresh medium now containing 10^{-6} M dexamethasone (Organon, Oss, The Netherlands). After a 24-hr incubation period, the cell culture medium was collected (Harvest 2) and replaced by fresh medium (without dexamethasone). After 24 hr, the cell culture medium was collected again (Harvest 3) and replaced once more by fresh medium (without dexamethasone), which was then harvested after another 24 hr-incubation period (Harvest 4). The harvested media were pooled and clarified by centrifugation for 10 min at 10,000 × *g*; the virus was then pelleted by centrifugation at 100,000 × *g* for 60 min, resuspended in 20 ml of NT buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4), sedimented again, and resuspended in 0.2 ml of NT buffer.

RNA-directed DNA polymerase assay. The virions were disrupted by mixing 75 μl of the virus suspension (in NT buffer) with 75 μl of a solution containing 100 mM Tris-HCl (pH 8.3) and in a reaction mixture containing, in a total volume of 100 μl, 0.1% Triton X-100, 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 0.5 mM $Mn(OOC\text{-}CH_3)_2$, 5 mM dithiothreitol, [*methyl*-^{3}H]dTTP (50 μCi/nmole per milliliter), 1.9 μM oligo$(dT)_{12\text{-}18}$, and 7.5 μM $(A)_n$. The reaction mixtures were incubated at 35° for 1 hr; the reaction was terminated by the addition of 100 μg of yeast RNA and 10 volumes of a solution containing 5% (w/w) trichloroacetic acid and 20 mM natrium pyrophos-

phate. Acid-insoluble material was collected on membrane filters, washed exhaustively with the trichloroacetic acid-pyrophosphate solution, then washed twice with ethanol, heat-dried, and counted in a toluene-based scintillant. Thus, the virion-associated RNA-directed DNA polymerase activity was measured with the synthetic homopolymer-oligomer $(A)_n \cdot (dT)_{12-18}$ as template-primer. $(A)_n \cdot (dT)_{12-18}$ can indeed be considered as an efficient template primer for oncornavirus RNA-directed DNA polymerase (22). Parallel reaction mixtures, intended to measure DNA-directed DNA polymerase activity, contained 7.5 μM $(dA)_n$ instead of $(A)_n$ as template. In contrast with the $(A)_n$-directed reaction, [*methyl*-^{3}H]dTMP incorporation in the $(dA)_n$-directed reaction never arose above background levels.

Cytotoxicity. Cytoxicity measurements were based on the number of cells (determined by Coulter counting) at 96 hr, that is, immediately after the fourth harvest of cell culture medium (see Induction of Retrovirus Particles).

Sucrose velocity gradient analysis. Virus suspensions (0.5 ml in NT buffer) were layered onto 15 ml of a 10%–60% (w/w) sucrose gradient (in NT buffer) and centrifuged for 16 hr at 25,000 rpm in a fixed-angle rotor (Type 30) of a Beckman L5-50 ultracentrifuge. Twenty-four fractions were collected from the bottom of the tubes and pooled in five sets (according to their density): 1.10–1.12 g/ml, 1.12–1.14 g/ml, 1.14–1.16 g/ml, 1.16–1.18 g/ml, and 1.18–1.20 g/ml. For all five sets of the sucrose gradient fraction pools, reverse transcriptase activity was determined as described above.

Electron microscopy. The pooled sucrose gradient fractions were sedimented onto membrane filters (25-nm pore size) by centrifugation at 40,000 rpm for 90 min. The filters were then fixed for 1 hr with 3% (w/w) glutaraldehyde in 0.1 M natrium cacodylate buffer (pH 7.4), washed with 7.5% sucrose in 0.1 M cacodylate, and post-fixed for 30 min in OsO_4 [1% (v/v) in 0.1 M cacodylate]. After dehydration with ethanol, the filters were embedded in Araldite (Ciba-Geigy, Basle, Switzerland). Sections were stained with lead citrate and examined with a Philips EM-201 electron microscope operating at 80 kV.

Incorporation into host cell DNA. Confluent BALB/3T3 cell monolayers in 60-mm Falcon plastic Petri dishes (approximately 10^6 cells/Petri dish) were incubated for 20 hr in the presence of either [5-^{125}I]dUrd (2.5 μCi/2.5 μg per milliliter per Petri dish) or *E*-5-[2-^{125}I-*vinyl*]dUrd (25 μCi/25 μg per milliliter per Petri dish). The cells were then washed with cold saline and lysed with 2% (w/w) sodium dodecyl sulfate 0.15 M NaCl, and 0.1 M EDTA, pH 8.2. The lysates were mixed with CsCl at a final density of 1.70 g/ml and centrifuged for 72 hr at 30,000 rpm in an MSE swinging bucket rotor (6 × 4.2 ml) of an MSE superspeed 65 ultracentrifuge. Five-drop fractions were collected from the bottom of the tubes, and assayed for radioactivity in a Packard Autogamma scintillation spectrometer, Type 5110.

RESULTS

To assess their ability as retrovirus inducers, all compounds were tested at several concentrations up to 400 μg/ml if not cytotoxic, or lower if cytotoxic (Tables 1 and 2).

In accord with previous observations (21, 23, 24), 5-iodo-dUrd appeared extremely efficient in stimulating the production of oncornavirus particles in BALB/3T3 cells, as monitored by the appearance of virus particle-bound $(A)_n \cdot$oligo(dT)-directed DNA polymerase ("reverse transcriptase") activity in the cell culture fluid (Table 1). This inducing effect was observed for varying concentrations of 5-iodo-dUrd, ranging from 0.04 to 40 μg/ml, but was most pronounced at 4 μg/ml. At this concentration, $(A)_n \cdot$oligo(dT)-directed DNA polymerase activity amounted to 50 times its background activity. At 40 μg/ml, 5-iodo-dUrd was somewhat less effective as an oncornavirus inducer, probably because of cytotoxicity (Table 2).

In addition to 5-iodo-dUrd, some other nucleoside analogues, viz., 5-iodo-dCyd, 5-bromo-dCyd, and 5-propynyloxy-dUrd, were found to stimulate the release of $(A)_n \cdot$oligo(dT)-directed DNA polymerase activity from BALB/3T3 cells, albeit at concentrations which were 100-fold (5-iodo-dCyd, 5-bromo-dCyd) to 10,000-fold (5-propynyloxy-dUrd) higher than those required for 5-iodo-dUrd (Table 1). The inducing effects of 5-iodo-dCyd and 5-bromo-dCyd probably result from their intracellular deamination to 5-iodo-dUrd and 5-bromo-dUrd.

Various other nucleoside analogues, including the selective antiherpes agents acycloguanosine (2), thymine arabinoside (3), 5-ethyluracil arabinoside (4), 5-propyl-dUrd (6), *E*-5-(2-bromovinyl)-dUrd (10), 5-methylthiomethyl-dUrd (8), 5-methylsulfonylmethyl-dUrd (9), and 5′-amino-5-iodo-2′,5′-ddUrd (13), failed to induce oncornavirus-associated DNA polymerase activity (Table 1). 5-Trifluoromethyl-dUrd and 5-vinyl-dUrd also failed to induce DNA polymerase activity, but, since these compounds were cytotoxic at concentrations as low as 0.04–0.4 μg/ml (Table 2), possible inducing ability may have been masked by toxicity.

That the $(A)_n \cdot$oligo(dT)-directed DNA polymerase activity detected in the BALB/3T3 cell culture fluids (Table 1) originated from oncornavirus (C-type virus) particles was ascertained by measuring the density of the enzyme-containing particles. Mouse C-type virus particles normally band at a density of 1.15–1.16 g/ml (25). This proved also to be the case for the DNA polymerase-containing particles induced by 5-iodo-dUrd and 5-propynyloxy-dUrd in BALB/3T3 cells (Fig. 1): when analyzed by sucrose density gradient ultracentrifugation, both the 5-iodo-dUrd- and 5-propynyloxy-dUrd-induced particles peaked at a gravity of 1.14–1.16 g/ml. In addition, the samples derived from 5-propynyloxy-dUrd-treated cells displayed a prominent band sedimenting at 1.10–1.12 g/ml. (The material banding at this density was not further characterized.) Parallel samples from untreated cells or cells which had been treated with *E*-5-(2-bromovinyl)-dUrd (4 μg/ml) displayed no enzyme activity in any of the sucrose gradient fractions. The particles that were released from 5-iodo-dUrd- and 5-propynyloxy-dUrd-treated BALB/3T3 cells and that banded in the gravity range of 1.14–1.16 g/ml (Fig. 1) were examined by electron microscopy: both the 5-iodo-dUrd- and 5-propynyloxy-dUrd-induced particles behaved morphologically as typical C-type virus particles (data not shown).

TABLE 1

Ability of different anti-herpes compounds to induce oncornavirus particles in BALB/3T3 cell cultures

Cells were incubated in the presence of the compounds for 24 hr and further incubated in the absence of compound for another 72 hr, at which time DNA polymerase activity was measured in the cell culture fluids (see Materials and Methods). The data represent mean values for two to six experiments. For 5-iodo-dUrd and the control group the range of individual values (six experiments) is indicated in parentheses. All values are expressed as net counts per min (minus background).

Compound	$(A)_n$·oligo(dT)-directed DNA polymerase activity: [*methyl*-^{3}H]dTMP incorporated at compound concentration of				
	0.04 μg/ml	0.4 μg/ml	4 μg/ml	40 μg/ml	400 μg/ml
			cpm		
Phosphonoacetate				2,180	397 (tox)[a]
Phosphonoformate				1,559	437 (tox)
Acycloguanosine		1,879	2,061	966 (tox)	
Adenine arabinoside				1,030 (tox)	1,167 (tox)
Uracil arabinoside				3,887	3,170
Thymine arabinoside			2,526	2,527	1,071
5-Ethyluracil arabinoside				4,903	1,103
5-Trifluoromethyl-dUrd	333 (tox)	192 (tox)	27 (tox)	143 (tox)	
5-Ethyl-dUrd			626	1,950 (tox)	2,386 (tox)
5-Propyl-dUrd			302	897	757
5-Propynyloxy-dUrd		2,129	2,126	5,913	52,596
5-Vinyl-dUrd		1,717 (tox)	2,280 (tox)	1,030 (tox)	
5-Ethynyl-dUrd		1,342	1,993 (tox)	2,143 (tox)	
E-5-(2-chlorovinyl)-dUrd			2,093	795	
E-5-(2-bromovinyl)-dUrd	3,128	2,644	2,669	2,605 (tox)	
E-5-(2-iodovinyl)-dUrd		2,378	1,614	1,760 (tox)	
5-Methylthiomethyl-dUrd				2,534	2,034
5-Methylsulfonylmethyl-dUrd			1,307	1,459	449 (tox)
5-Bromo-dCyd		3,011	45,671	159,360	194,117
5-Iodo-dCyd		1,584	17,475	131,701	
5′-Amino-5-iodo-2′,5′-ddUrd			109	281	1,269
5-Iodo-dUrd	17,105 (16,136–18,073)	71,884 (40,884–86,527)	91,210 (tox) (88,254–95,480)	56,952 (tox) (36,396–79,977)	
Control					
With dexamethasone	2,085 (316–5,941)				
Without dexamethasone	1,387 (419–3,833)				

[a] Refers to a reduction of ≥50% in cell number, as measured at the 4th day, when the supernatant fluids were harvested for DNA polymerase activity determinations. The exact percentages are presented in Table 2.

The selective antiherpes activity of compounds like acycloguanosine (2, 15), thymine arabinoside (3, 15), *E*-5-(2-bromovinyl)-dUrd (10, 15), and 5′-amino-5-iodo-2′,5′-ddUrd (13, 15) mainly depends on their phosphorylation by the herpesvirus-induced thymidine kinase. As a consequence, these compounds are converted to the triphosphate in herpesvirus-infected cells to a much greater extent than in uninfected cells. Hence, the inability of *E*-5-(2-bromovinyl)-dUrd and congeners to induce the release of C-type virus particles from BALB/3T3 might possibly result from inadequate phosphorylation. To resolve this point, additional experiments were designed in which the cells were first infected with HSV-1 (strain KOS) and subsequently exposed to *E*-5-(2-bromovinyl)-dUrd (Fig. 2). In these experiments, 5-iodo-dUrd served as the reference material, and both 5-iodo-dUrd and *E*-5-(2-bromovinyl)-dUrd were used at a concentration (4 μg/ml) that was considered optimal for the induction of oncornavirus particles (Table 1). While HSV-infected BALB/3T3 cells continued to release oncornavirus particles upon 5-iodo-dUrd treatment, no such release was observed for HSV-infected *E*-5-(2-bromovinyl)-dUrd-treated BALB/3T3 cells, irrespective of the multiplicity at which they had been infected with HSV-1 (KOS) (Fig. 2). Thus, neither HSV-infected nor uninfected BALB/3T3 cells appear to synthesize oncornavirus particles in response to *E*-5-(2-bromovinyl)-dUrd.

Several lines of evidence (see Discussion) indicate that, if a given nucleoside analogue were to induce the production of endogenous oncornaviruses, it should be incorporated into DNA (18). Since *E*-5-(2-bromovinyl)-dUrd does not activate oncornavirus synthesis, one may wonder whether it is incorporated into DNA. This possibility was directly explored with a radiolabeled analogue of *E*-5-(2-bromovinyl)-dUrd, namely *E*-5-[2-^{125}I-*vinyl*]dUrd. BALB/3T3 cells were exposed for 20 hr to either 25 μg (25 μCi) or 2.5 μg (2.5 μCi) of *E*-5-[2-^{125}I-*vinyl*]dUrd/ml; the cell lysates were then analyzed by CsCl density gradient ultracentrifugation (Fig. 3). Under conditions in which high amounts of [5-^{125}I]dUrd were incorporated into BALB/3T3 DNA, no such incorporation was apparent for *E*-5-[2-^{125}I-*vinyl*]dUrd, whether it was applied at 2.5 μg (2.5 μCi)/ml (data not shown) or 25 μg (25 μCi)/ml (Fig. 3). On the basis of the counts

TABLE 2

Toxicity of different antiherpes compounds for BALB/3T3 cell cultures

Cells were incubated in the presence of the compounds for 24 hr and further incubated in the absence of compound for another 72 hr, at which time cell number was determined by Coulter counting. The data represent mean values for two to six experiments.

Compound	Cell no. (% of control) at compound concentration of				
	0.04 μg/ml	0.4 μg/ml	4 μg/ml	40 μg/ml	400 μg/ml
Phosphonoacetate				109	44
Phosphonoformate				107	48
Acycloguanosine		76	77	35	
Adenine arabinoside			107	34	26
Uracil arabinoside				105	108
Thymine arabinoside			105	113	88
5-Ethyluracil arabinoside				125	92
5-Trifluoromethyl-dUrd	21	3.8	3.6	3.0	
5-Ethyl-dUrd				49	30
5-Propyl-dUrd				120	77
5-Propynyloxy-dUrd				109	85
5-Vinyl-dUrd		22	4.3	5.3	
5-Ethynyl-dUrd			31	33	
E-5-(2-chlorovinyl)-dUrd			100	70	
E-5-(2-bromovinyl)-dUrd		93	65	37	
E-5-(2-iodovinyl)-dUrd		98	69	23	
5-Methylthiomethyl-dUrd				111	96
5-Methylsulfonylmethyl-dUrd			100	64	42
5-Bromo-dCyd			124	90	81
5-Iodo-dCyd				82	73
5′-Amino-5-iodo-2′,5′-ddUrd			101	121	92
5-Iodo-dUrd	108	72	43	30	
Control					
With dexamethasone	110				
Without dexamethasone	100				

obtained for the radioactivity input and output (Fig. 3), one may deduce that the extent at which *E*-5-[2-^{125}I-*vinyl*]dUrd was incorporated into BALB/3T3 cell DNA could not be greater than 0.1% of the extent of [5-^{125}I]-dUrd incorporation.

DISCUSSION

Present evidence strongly suggests that incorporation into DNA is required for the activation of oncornaviruses by nucleoside analogues such as 5-iodo-dUrd and 5-bromo-dUrd (18): (a) virus induction does not occur if DNA synthesis is blocked by cytosine arabinoside; (b) similarly, no virus is induced if the incorporation of 5-iodo- or 5-bromo-dUrd is prevented by simultaneous treatment with thymidine; (c) virus induction is potentiated if incorporation of the nucleoside analogues is enhanced by simultaneous treatment with 5-fluoro-dUrd; and, finally, (d) virus activation is enhanced if the cells that have been treated with suboptimal doses of 5-iodo-dUrd or 5-bromo-dUrd are subsequently exposed to visible light or X-ray irradiation (two procedures which are

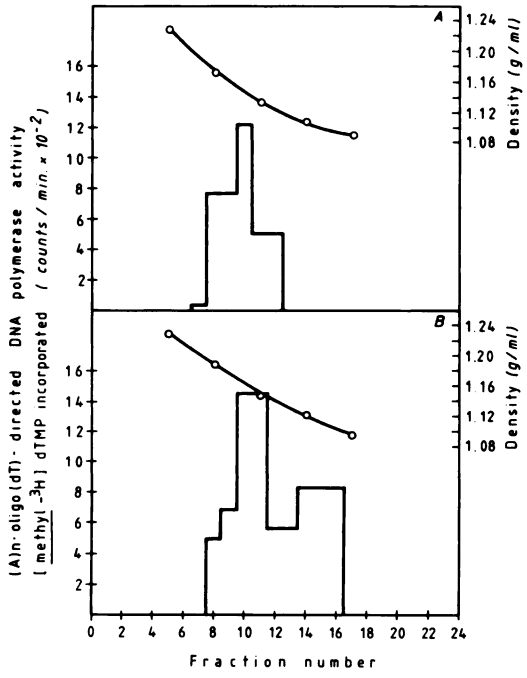

FIG. 1. *Sucrose velocity gradient analysis of (A)$_n$·oligo(dT)-directed DNA polymerase-containing particles released from BALB/3T3 cells after treatment with either 5-iodo-dUrd (4 μg/ml) (panel A) or 5-propynyloxy-dUrd (400 μg/ml) (panel B)*

known specifically to affect those DNA regions that contain incorporated nucleoside analogues).

Thus, those compounds that activate the release of oncornavirus particles may be assumed to be incorporated into DNA. This rule certainly applies to 5-iodo-dUrd, 5-bromo-dUrd, 5-iodo-dCyd, and 5-bromo-dCyd, and may also be extended to 5-propynyloxy-dUrd (Table 1; Fig. 1). However, it has not yet been demonstrated directly whether 5-propynyloxy-dUrd is incorporated into DNA. For those compounds that do not induce oncornavirus synthesis, one may postulate that they are not incorporated into DNA or, if incorporated, that they do not stimulate the expression of endogenous oncornavirus genes. One of the noninducers, *E*-5-(2-iodovinyl)-dUrd, was tested for its incorporation into BALB/3T3 cell DNA, and, as shown in Fig. 3, it was not incorporated to a significant extent.

Although activation of oncornavirus synthesis by a nucleoside analogue may be interpreted as proof of its incorporation into DNA, the reverse does not necessarily hold true. Indeed, an analogue that is incorporated into DNA may or may not act as an inducer of oncornavirus synthesis. 5-Iodo-dUrd and 5-bromo-dUrd do so, and 5-iodo-dCyd and 5-bromo-dCyd also act as virus inducers, probably after they have been converted intracellularly to their 2′-deoxyuridine counterparts. However, 5-ethyl-dUrd does not activate the release of C-type virus particles, as shown here (Table 1) and previously (19), although there is little doubt that 5-ethyl-dUrd can be incorporated into cellular DNA (26). 5-Trifluoromethyl-dUrd and 5-vinyl-dUrd are two other examples of nucleoside analogues that are incorporated into cellular DNA (27, 28). Neither 5-trifluoromethyl-dUrd nor 5-vi-

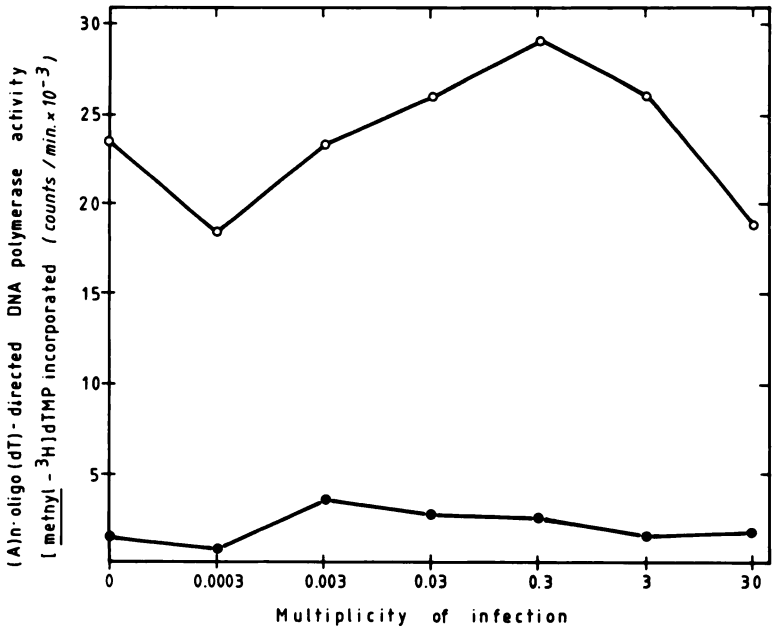

FIG. 2. *Effects of 5-iodo-dUrd (○——○) and E-5-(2-bromovinyl)-dUrd (●——●), both at 4μg/ml, on the release of oncornavirus particles from BALB/3T3 cells infected with HSV-1 (strain KOS) at different multiplicities of infection (plaque-forming units per cell)*

nyl-dUrd proved capable of inducing oncornavirus production (Table 1). However, these compounds were fairly toxic to BALB/3T3 cells (Table 2), and it is possible that their C-type virus-inducing potentials were masked by cytotoxicity. Acycloguanosine and 5′-amino-5-iodo-2′,5′-ddUrd represent yet two other examples of nucleoside analogues that are incorporated into DNA, although this incorporation is restricted to HSV-infected (or transformed) cells (29, 30). Although the former is incorporated terminally at the 3′-ends of growing DNA chains and acts as a chain terminator (29), the latter is incorporated internally and produces extensive DNA breaks due to the presence of the labile phosphoramidate (P—N) bonds (30). Neither acycloguanosine nor 5′-amino-5-iodo-2′,5′-ddUrd led to the induction of endogenous oncornaviruses (Table 1).

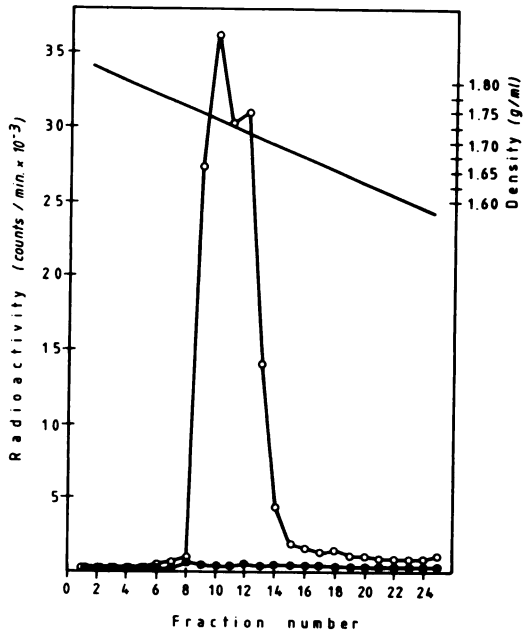

FIG. 3. *CsCl equilibrium gradient analysis of DNA of BALB/3T3 cells treated for 20 hr with either [5-^{125}I]dUrd (2.5 μCi/2.5 μg per milliliter) (○——○) or E-5-[2-^{125}I-vinyl]dUrd (25 μCi/25 μg per milliliter) (●——●)*

Acycloguanosine, 5′-amino-5-iodo-2′,5′-ddUrd, and several other nucleoside analogues such as *E*-5-(2-bromovinyl)-dUrd are preferentially phosphorylated by HSV-encoded thymidine kinase. One could argue, therefore, that these compounds, while inefficient as oncornavirus inducers in normal BALB/3T3 cells, might be more successful inducers in HSV-infected cells. This possibility was examined with *E*-5-(2-bromovinyl)-dUrd as probe, but, as shown in Fig. 2, HSV infection did not endow *E*-5-(2-bromovinyl)-dUrd with oncornavirus-inducing ability. However, whether *E*-5-(2-bromovinyl)-dUrd was phosphorylated in the HSV-infected BALB/3T3 cells was not assessed.

The fact that the 5-(2-halogenovinyl) derivatives *E*-5-(2-bromovinyl)-dUrd, *E*-5-(2-chlorovinyl)-dUrd, and *E*-5-(2-iodovinyl)-dUrd—unlike the 5-halogenated derivatives 5-iodo-dUrd and 5-bromo-dUrd—failed to induce C-type virus particles from BALB/3T3 cells could be considered as an attribute of safety. Indeed, C-type virus particles, even when induced in nontransformed cells, are potentially carcinogenic, as has been demonstrated for the endogenous virus induced by 5-iodo-dUrd in C57BL/6 mouse embryo cells (17).

From the data presented in Table 1, one could calculate the minimal inductive doses required to induce the release of C-type virus particles from BALB/3T3 cells; and from the data presented in Table 2, one could determine the minimal toxic doses required to reduce cell proliferation. When these minimal inductive doses or

minimal toxic doses were compared with the minimal effective doses that were found inhibitory to HSV-1 replication (4, 8, 9, 14, 15), it appeared that several compounds, viz., acycloguanosine, thymine arabinoside, 5-ethyluracil arabinoside, 5-propyl-dUrd, *E*-5-(2-chlorovinyl)-dUrd, *E*-5-(2-bromovinyl)-dUrd, and *E*-5-(2-iodovinyl)-dUrd displayed a rather high selectivity index as antiherpes agents (ratio B:A in Table 3). However, 5-trifluoromethyl-dUrd and 5-iodo-dUrd were negatively selective in that their minimal inductive or toxic doses were lower than the antiviral one (Table 3). For the 5-halogenated 2′-deoxycytidines, the minimal inductive doses were 5-fold (5-bromo-dCyd) to 35-fold (5-iodo-dCyd) higher than the antiviral doses, and for 5-propynyloxy-dUrd it was even 70-fold higher. Thus, on the basis of the ratios of the minimal oncornavirus-inductive doses to the minimal antiherpesvirus doses, acycloguanosine, thymine arabinoside, 5-propyl-dUrd, and the *E*-5-(2-halogenovinyl)-2′-deoxyuridines emerged as the most selective antiherpes agents. The same compounds have also proven their selectivity in previous studies in which other criteria, i.e., microscopic alteration of cell morphology or inhibition of cellular DNA synthesis, served as parameters of cytotoxicity (14, 15).

TABLE 3

Specificity indices of different anti-herpes compounds

Compound	Minimal effective dose required to inhibit HSV-1 replication in primary rabbit kidney cell cultures[a] (A)	Minimal inductive dose required to induce oncornavirus release from BALB/3T3 cells[b] or, if cytotoxic, minimal toxic dose for BALB/3T3 cells[c] (B)	Ratio B:A
	μg/ml	*μg/ml*	
Phosphonoacetate	13	400[c]	30
Phosphonoformate	13	400[c]	30
Acycloguanosine	0.04	10[c]	250
Adenine arabinoside	7	10[c]	1.4
Uracil arabinoside	43	>400[b]	>9.3
Thymine arabinoside	0.25	>400[b]	>1600
5-Ethyluracil arabinoside	2	>400[b]	>200
5-Trifluoromethyl-dUrd	0.7	<0.04[c]	<0.06
5-Ethyl-dUrd	0.5	40[c]	80
5-Propyl-dUrd	0.6	>400[b]	>670
5-Propynyloxy-dUrd	1.4	100[b]	70
5-Vinyl-dUrd	0.018	<0.4[c]	<22
5-Ethynyl-dUrd	0.6	<4[c]	<7
E-5-(2-chlorovinyl)-dUrd	0.02	>40[b]	>2000
E-5-(2-bromovinyl)-dUrd	0.008	10[c]	1250
E-5-(2-iodovinyl)-dUrd	0.012	10[c]	830
5-Methylthiomethyl-dUrd	6	>400[b]	>70
5-Methylsulfonylmethyl-dUrd	4	100[c]	25
5-Bromo-dCyd	0.2	1[b]	5
5-Iodo-dCyd	0.06	2[b]	35
5′-Amino-5-iodo-2′,5′-ddUrd	26	>400[c]	>15
5-Iodo-dUrd	0.13	0.04[b]	0.3

[a] As monitored by virus-induced cytopathogenicity (CPE); minimal effective dose corresponds to the dose inhibiting viral CPE by 50%. Most data are taken from ref. 15, except those for 5-ethyluracil arabinoside (ref. 4), 5-methylthiomethyl-dUrd (ref. 8), 5-methylsulfonylmethyl-dUrd (ref. 9), and 5-bromo-dCyd (ref. 14).

[b] As monitored by RNA-directed DNA polymerase activity (Table 1); minimal inductive dose corresponds to incorporation of approximately 10,000 cpm of [*methyl*-^{3}H]dTMP.

[c] As monitored by reduction in cell number (Table 2); minimal toxic dose corresponds to the dose reducing cell number by approximately 50%.

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Send reprint requests to: Dr. Erik De Clercq, Department of Human Biology, Division of Microbiology, Rega Institute for Medical Research, Katholiecke Universiteit Leuven, B-3000 Leuven, Belgium.