Antiviral activity of the 3'-amino derivative of (*E*)-5-(2-bromovinyl)-2'-deoxyuridine

Erik DE CLERCQ,* Johan DESCAMPS,*†‡ Jan BALZARINI,* Toshikazu FUKUI*§
and H. S. ALLAUDEEN†||

**Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and †Department of Pharmacology, Yale University of Medicine, Sterling Hall of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.*

(Received 11 October 1982/Accepted 14 January 1983)

3'-NH₂-BV-dUrd, the 3'-amino derivative of (*E*)-5-(2-bromovinyl)-2'-deoxyuridine, was found to be a potent and selective inhibitor of herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) replication. 3'-NH₂-BV-dUrd was about 4–12 times less potent but equally selective in its anti-herpes activity as BV-dUrd. Akin to BV-dUrd, 3'-NH₂-BV-dUrd was much less inhibitory to herpes simplex virus type 2 than type 1. It was totally inactive against a thymidine kinase-deficient mutant of HSV-1. The 5'-triphosphate of 3'-NH₂-BV-dUrd (3'-NH₂-BV-dUTP) was evaluated for its inhibitory effects on purified herpes viral and cellular DNA polymerases. Among the DNA polymerases tested, HSV-1 DNA polymerase and DNA polymerase α were the most sensitive to inhibition by 3'-NH₂-BV-dUTP (K_i values 0.13 and 0.10 μ M, respectively). The K_m/K_i ratio for DNA polymerase α was 47, as compared with 4.6 for HSV-1 DNA polymerase. Thus, the selectivity of 3'-NH₂-BV-dUrd as an anti-herpes agent cannot be ascribed to a discriminative effect of its 5'-triphosphate at the DNA polymerase level. This selectivity most probably resides at the thymidine kinase level. 3'-NH₂-BV-dUrd would be phosphorylated preferentially by the HSV-1-induced thymidine kinase (K_i 1.9 μ M, as compared with >200 μ M for the cellular thymidine kinase), and this preferential phosphorylation would confine the further action of the compound to the virus-infected cell.

During the past few years we have witnessed the development of several nucleoside analogues which show great promise as selective anti-herpes agents.

Abbreviations used: BV-dUrd, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine; 3'-NH₂-BV-dUrd, (*E*)-5-(2-bromovinyl)-3'-amino-2',3'-dideoxyuridine; BV-Ara-U, 1- β -D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil; 3'-NH₂-BV-dUMP and 3'-NH₂-BV-dUTP, the 5'-monophosphate and 5'-triphosphate of 3'-NH₂-BV-dUrd; 5-iodo-dUrd, 5-iodo-2'-deoxyuridine; FI-Ara-C, 2'-fluoro-5-iodo-1- β -D-arabinofuranosylcytosine; HSV-1, HSV-2, herpes simplex virus types 1 and 2; VZV, varicella-zoster virus; ID₅₀, dose required for 50% inhibition.

‡ Present address: Smith-Kline-RIT, Rue de l'Institut 89, B-1330 Rixensart, Belgium.

§ Present address: Faculty of Pharmaceutical Sciences, Osaka University, 133-1 Yamada-Kami, Suita, Osaka, Japan.

|| Present address: Smith Kline and French Laboratories, 1500 Spring Garden Street, Philadelphia, PA 19101, U.S.A.

Some of these compounds, i.e. acyclovir [9-(2-hydroxyethoxymethyl)guanine], FI-Ara-C and BV-dUrd have already been submitted to clinical trials in patients with severe HSV-1, HSV-2 or VZV infections.

BV-dUrd and the other 5-(2-halogenovinyl)-2'-deoxyuridines have emerged as the most potent and most selective anti-HSV-1 agents among a wide variety of 5-substituted 2'-deoxyuridines (De Clercq, 1980). They inhibit the replication of HSV-1 and VZV at a concentration of about 0.01 μ g/ml, which is three to four orders of magnitude lower than the concentration required to inhibit normal cell metabolism (De Clercq *et al.*, 1980b, 1982a). However, HSV-2 is much less susceptible to the inhibitory effects of the 5-(2-halogenovinyl)-2'-deoxyuridines, since BV-dUrd and its congeners do not inhibit the replication of HSV-2 unless their concentration is increased to 1–2 μ g/ml, that is 100–200-fold the inhibitory concentration for HSV-1 (De Clercq *et al.*, 1980a,b).

In attempts to develop other compounds with selective anti-herpes activity and, if at all possible, greater potency towards HSV-2, several BV-dUrd analogues were prepared with modifications at either C-2, C-3 or C-5 of the sugar moiety, i.e. arabinofuranose, 2-deoxyxylofuranose, 3-amino-2,3-dideoxyribose, 3-azido-2,3-dideoxyribose, 3-chloro-2,3-dideoxyribose, 5-amino-2,5-dideoxyribose, 5-azido-2,5-dideoxyribose, 5-chloro-2,5-dideoxyribose, 5-bromo-2,5-dideoxyribose, and 5-iodo-2,5-dideoxyribose. Of this series of BV-dUrd derivatives, only four compounds [BV-Ara-U, 3'-NH₂-BV-dUrd, (*E*)-5-(2-bromovinyl)-3'-azido-2',3'-dideoxyuridine and (*E*)-5-(2-bromovinyl)-5'-azido-2',5'-dideoxyuridine] exhibited a marked anti-herpes potency (Busson *et al.*, 1981).

The anti-herpes activity of BV-Ara-U and the molecular basis for its anti-herpes action have been dealt with in a previous report (Descamps *et al.*, 1982). The present study is addressed to the anti-herpes activity of 3'-NH₂-BV-dUrd and its mechanism of action. The inhibitory effects of 3'-NH₂-BV-dUrd on herpes virus replication were evaluated in several cell cultures, and a variety of viral and cellular DNA polymerases were compared for their sensitivity to inhibition by 3'-NH₂-BV-dUTP.

Part of this study has been presented at the 12th International Congress of Chemotherapy, Florence, Italy, July 1981 (De Clercq *et al.*, 1982b).

Materials and methods

Cells

The cell cultures used were: PRK, primary rabbit kidney; HSF, human skin fibroblasts (strain VGS); ESM, human embryonic skin-muscle fibroblasts; HEL, human embryonic lung fibroblasts; T-21, human trisomic-21 fibroblasts (strain LR); BS-C-1, a simian epithelial cell line originated from African green monkey kidney; FL, feline lung fibroblasts; BALB/3T3, murine fibroblasts derived from BALB/c mouse embryos. The T-21 cell strain LR was provided by Dr. J. J. Cassiman (Division of Human Genetics, University of Leuven, Belgium); BS-C-1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.); the FL cell line was obtained from Flow Laboratories (Irvine, Scotland, U.K.) and the BALB/3T3 cell line was provided by Dr. S. A. Aaronson (National Cancer Institute, Bethesda, MD, U.S.A.). The other cell lines originated from our own laboratory. The cells were grown in Eagle's minimal essential medium supplemented with 10% (v/v) foetal bovine serum.

Compounds

3'-NH₂-BV-dUrd and BV-Ara-U were synthesized from BV-dUrd as the starting material (Busson

et al., 1981). BV-dUrd itself was synthesized following a slight modification of the procedure described by Jones *et al.* (1979). 5-Iodo-dUrd was obtained from Ludeco, Brussels, Belgium.

3'-NH₂-BV-dUrd was converted to the corresponding 5'-monophosphate (3'-NH₂-BV-dUMP) by POCl₂/PO(OEt)₃ (Yoshikawa *et al.*, 1967) (yield 68%). 3'-NH₂-BV-dUMP was further converted to the 5'-triphosphate (3'-NH₂-BV-dUTP) via the phosphorimidazolate (Hoard & Ott, 1965; Hecht & Kozarich, 1973). The end product was purified on DEAE-cellulose and obtained in 44% yield.

Viruses

The origin of the HSV-1 and HSV-2 strains has been described previously (De Clercq *et al.*, 1980b). The VZV strain VFW 505 was a clinical isolate obtained from an adult female patient with severe chickenpox.

Antiviral assays

The procedure for measuring HSV-induced cytopathogenicity in PRK (De Clercq *et al.*, 1980b) and other (De Clercq, 1982) cell cultures, and HSV growth in PRK cells (De Clercq *et al.*, 1979) have been described. The methods for determining the cytopathogenicity of cell-free VZV and focus-forming ability of cell-associated VZV have also been described (De Clercq *et al.*, 1982a).

Cytotoxicity

We used the inhibition of host cell DNA synthesis as a parameter of cytotoxicity. This DNA synthesis was monitored by the incorporation of 2'-deoxy[1',2'-³H]uridine into DNA of uninfected PRK cells (De Clercq *et al.*, 1980b).

Isolation of cellular and viral thymidine kinase, and thymidine kinase assays

The procedures for the isolation of human cytosol, HSV-1 and HSV-2 thymidine kinases have been described previously (Cheng & Ostrander, 1976; Lee & Cheng, 1976). The *K_i* values were determined by the procedure of Cheng & Prusoff (1973). The thymidine kinase activity of PRK cells was measured as described previously for the thymidine kinase activity of human and murine tumour cell lines (Balzarini *et al.*, 1982).

Isolation of cellular and viral DNA polymerases, and DNA polymerase assays

The procedures for isolation of the DNA polymerases α and β , HSV-1 DNA polymerase and Epstein-Barr virus DNA polymerase, and for the assays of these enzymes have been described previously (Allaudeen *et al.*, 1981; Allaudeen & Rani, 1982). The *K_i* and *K_m* values of 3'-NH₂-BV-dUTP were determined as previously estab-

lished for BV-dUTP (Allaudeen *et al.*, 1981). The K_i was defined as the concentration of compound that under the conditions used gave 50% inhibition of the DNA polymerase reaction.

Results and discussion

Potency and selectivity of 3'-NH₂-BV-dUrd as an anti-herpes agent

The 3'-amino derivative of BV-dUrd inhibited the cytopathogenicity of HSV-1, HSV-2 and VZV at an ID₅₀ which was 4–25-fold higher than that of BV-dUrd itself (Table 1). The ID₅₀ of 3'-NH₂-BV-dUrd for HSV-1 (strain KOS) remained fairly constant for a wide variety of cell lines (Table 2). 3'-NH₂-BV-dUrd proved less potent as an HSV-1 inhibitor than BV-dUrd in all cell lines, except feline lung cells, where the relative order of potency was reversed. 3'-NH₂-BV-dUrd was the least efficient in BS-C-1 cells, although it was still more effective in these cells than BV-Ara-U (Table 2).

Whereas 5-iodo-dUrd inhibited HSV-2 replication at an ID₅₀ which was only two-fold higher than its ID₅₀ for HSV-1, 3'-NH₂-BV-dUrd, BV-Ara-U and BV-dUrd inhibited HSV-2 at an ID₅₀ which was 125–350-fold higher than the ID₅₀ for HSV-1 (Table 1). In analogy with BV-dUrd (De Clercq *et al.*, 1980b), 3'-NH₂-BV-dUrd and BV-Ara-U could be considered as useful markers to discriminate between HSV-1 and HSV-2.

The specificity of 3'-NH₂-BV-dUrd and BV-Ara-U as inhibitors of HSV-1 was further attested by their failure to inhibit the replication of vaccinia virus (Table 1). This contrasted with 5-iodo-dUrd, which inhibited vaccinia virus at an ID₅₀ that was only two-fold higher than the ID₅₀ for HSV-1. As could be expected from truly selective anti-herpes agents, neither 3'-NH₂-BV-dUrd nor BV-Ara-U had an adverse effect on normal cell metabolism, as monitored by 2'-deoxy[1',2'-³H]uridine incorporation into host cell DNA, unless they were used at a 1000–2000-fold higher concentration than that required to inhibit HSV-1 replication (Table 1). Again, this contrasted with 5-iodo-dUrd, which inhibited 2'-deoxy[1',2'-³H]uridine incorporation at a dose that corresponded closely to its ID₅₀ for HSV-1.

While substitution of the sugar moiety of BV-dUrd by arabinofuranose, 3-amino-2,3-dideoxyribose, or any other modified 2-deoxyribose (results not shown), uniformly led to a decreased activity against HSV-2, substitution of arabinofuranose for 2-deoxyribose brought about a 7-fold increase in anti-VZV activity (Table 1). Depending on the VZV strain studied, the increase in activity gained upon the conversion of BV-dUrd to BV-Ara-U varied 2–10-fold (S. Shigeta, M. Ogata & E. De Clercq, unpublished work). These observations have recently been confirmed by Machida *et al.* (1982). As reflected by the ID₅₀ values obtained in the present

Table 1. Antiviral activity of 3'-NH₂-BV-dUrd in PRK or ESM cells

ID₅₀ is the dose required to reduce virus-induced cytopathogenicity or host cell metabolism by 50%. The ID₅₀ values for HSV-1 and HSV-2 represent mean values for three laboratory strains (KOS, F and McIntyre) of HSV-1 and three laboratory strains (Lyons, G and 196) of HSV-2. The VZV strain used was a clinical isolate (VFW 505) and the ID₅₀ values reported for this strain represent the mean values of three separate experiments. All antiviral tests were performed in PRK cells except for VZV which was assayed in ESM cells. The ID₅₀ for cell metabolism refers to the concentration of compound required to inhibit by 50% the incorporation of 2'-deoxy[1',2'-³H]uridine into DNA of uninfected PRK cells.

Compound	ID ₅₀ (μg/ml) for:				
	HSV-1	HSV-2	VZV	Vaccinia virus	Cell metabolism
3'-NH ₂ -BV-dUrd	0.1	25	0.03	200	200
BV-Ara-U	0.1	35	0.001	200	100
BV-dUrd	0.008	1	0.007	7	20
5-Iodo-dUrd	0.15	0.3	0.4	0.3	0.25

Table 2. Inhibitory effect of 3'-NH₂-BV-dUrd on HSV-1 (strain KOS) in different cell cultures

ID₅₀ is the dose required to reduce virus-induced cytopathogenicity by 50%. For BV-Ara-U, BV-dUrd and 5-iodo-dUrd the ID₅₀ values are taken from De Clercq (1982).

Compound	Cell culture ...	ID ₅₀ (μg/ml)							
		PRK	HSF	ESM	HEL	T-21	BS-C-1	FL	BALB/3T3
3'-NH ₂ -BV-dUrd		0.07	0.2	0.07	0.2	0.02	4	0.07	0.07
BV-Ara-U		0.1	0.02	0.007	0.01	0.01	200	0.04	0.06
BV-dUrd		0.007	0.01	0.007	0.01	0.005	0.02	0.2	0.007
5-Iodo-dUrd		0.15	0.4	0.1	0.1	0.08	0.4	2	0.1

(Table 1) and previous (S. Shigeta, M. Ogata & E. De Clercq, unpublished work) studies, 3'-NH₂-BV-dUrd was slightly less active as a VZV inhibitor than was BV-dUrd. Its potency against VZV would correspond to that of FI-Ara-C (Machida *et al.*, 1982), which means significantly higher than that of acyclovir (De Clercq *et al.*, 1982a; Machida *et al.*, 1982).

The results presented for VZV in Table 1 concern viral cytopathogenicity. In a focus-reduction assay, based on focus formation by cell-associated VZV in ESM cells (results not shown), 3'-NH₂-BV-dUrd effected a 50% reduction at a concentration of 0.03 µg/ml; thus, identical with the ID₅₀ required for suppression of viral cytopathogenicity (Table 1). For BV-Ara-U, the ID₅₀ required to suppress VZV focus formation was also identical to the ID₅₀ for suppression of viral cytopathogenicity (0.001 µg/ml).

On the basis of its inhibitory effects on viral cytopathogenicity, 3'-NH₂-BV-dUrd could be considered as a potent inhibitor of HSV-1 and VZV replication. This assumption was borne out by virus growth reduction experiments, where virus yield was measured at different times after HSV-1 (KOS)-infected PRK cells had been exposed to varying concentrations of 3'-NH₂-BV-dUrd (Fig. 1). The

inhibitory effects of 3'-NH₂-BV-dUrd on virus growth were directly compared with those of BV-dUrd. At 0.01 µg/ml, neither BV-dUrd nor 3'-NH₂-BV-dUrd were effective. At 0.1 µg/ml, BV-dUrd achieved a greater virus yield reduction than did 3'-NH₂-BV-dUrd but, at higher concentrations (1 and 10 µg/ml), 3'-NH₂-BV-dUrd reduced the virus yield to a greater extent than did BV-dUrd (Fig. 1).

Role of viral thymidine kinase in antiviral activity of 3'-NH₂-BV-dUrd

Neither 3'-NH₂-BV-dUrd nor its predecessors, BV-Ara-U and BV-dUrd, were active against a TK⁻ (thymidine kinase-deficient) mutant of HSV-1 (strain B2006) (results not shown), which suggests that their antiviral activity depends on a specific phosphorylation by the HSV-1-induced thymidine kinase. The affinity of 3'-NH₂-BV-dUrd, BV-Ara-U and BV-dUrd for HSV-1 thymidine kinase is much higher than their affinity for the cellular or cytosol thymidine kinase, as reflected by the inhibition constants (*K_i*) of these nucleoside analogues for the different types of thymidine kinases (Table 3). The differential affinity of 3'-NH₂-BV-dUrd, BV-Ara-U and BV-dUrd for thymidine kinases of various (i.e.

Fig. 1. Inhibition of the growth of HSV-1 (strain KOS) by BV-dUrd (a) and 3'-NH₂-BV-dUrd (b) in PRK cells. Virus input: 4.5 log₁₀ plaque-forming units/petri dish (multiplicity of infection 0.03 plaque-forming units/cell). BV-dUrd and 3'-NH₂-BV-dUrd were added at different concentrations immediately after virus adsorption. Virus yield was determined at 1 h, and 1, 2 and 3 days after virus infection by plaque formation in VERO cell cultures. Concentrations of BV-dUrd and 3'-NH₂-BV-dUrd were: ▽, 10 µg/ml; □, 1 µg/ml; △, 0.1 µg/ml; ○, 0.01 µg/ml; ●, 0 µg/ml.

Table 3. Inhibition constants of 3'-NH₂-BV-dUrd for various kinds of thymidine kinase

Compound	Enzyme source	...	K _i (μM)			
			PRK	Human cytosol	HSV-1	HSV-2
3'-NH ₂ -BV-dUrd			>200	>50*	1.9*	>30*
BV-Ara-U			>400	>100†	0.94†	54.67†
BV-dUrd			>400	>100‡	0.24‡	4.24‡

* Unpublished data (Y.-C. Cheng & E. De Clercq).
† Data taken from Cheng *et al.* (1981b).
‡ Data taken from Cheng *et al.* (1981a).

Fig. 2. Inhibition of DNA polymerases by 3'-NH₂-BV-dUTP

Percentage of activity remaining is plotted against increasing concentrations of 3'-NH₂-BV-dUTP. The enzyme activities were measured by [³H]dTTP incorporation, with activated DNA as template. The concentrations of [³H]dTTP used in these experiments were two to three times the K_m values of the corresponding enzymes; the other three triphosphates were in excess. DNA polymerases: O, HSV-1 DNA polymerase; ●, DNA polymerase α; □, DNA polymerase β; Δ, Epstein-Barr virus DNA polymerase.

viral and cellular) origin may not only account for their selectivity as anti-herpes agents, but also contribute to the differences in the activities of these nucleoside analogues against various herpes viruses. For example, BV-dUrd, BV-Ara-U and 3'-NH₂-BV-dUrd are active against HSV-2 only at those concentrations that are 100–1000-fold higher than the concentrations required to inhibit HSV-1 replication, and this discriminating behaviour is reflected by their affinities for HSV-1 and HSV-2 thymidine kinase (Table 3).

Sensitivity of viral and cellular DNA polymerases to inhibition of 3'-NH₂-BV-dUTP

The ability of 3'-NH₂-BV-dUTP to inhibit the utilization of dTTP by viral and cellular DNA

Fig. 3. Effect of 3'-NH₂-BV-dUTP on HSV-1 DNA polymerase reaction in the presence of different concentrations of [³H]dTTP, with activated DNA as template O, No inhibitor; □, 0.2 μM-3'-NH₂-BV-dUTP; ●, 1 μM-3'-NH₂-BV-dUTP.

polymerase is depicted in Fig. 2. The DNA polymerases used in these assays were free of cross-contaminants and all assays were performed under conditions optimal for the individual enzymes. The concentration of [³H]dTTP was maintained at two to three times the K_m value for the corresponding enzyme. Fig. 2 shows that both the HSV-1 DNA polymerase and cellular DNA polymerase α were the most sensitive to inhibition by 3'-NH₂-BV-dUTP. DNA polymerase β was less sensitive, whereas Epstein-Barr virus DNA polymerase was insensitive, even at 20 μM-3'-NH₂-BV-dUTP. Preliminary results obtained for HSV-2 DNA polymerase indicate that this enzyme behaves similarly to DNA polymerase β.

Fig. 4. Effect of 3'-NH₂-BV-dUTP on DNA polymerase α reaction in the presence of different concentrations of [³H]dTTP, with activated DNA as template

Other assay conditions were optimal for the DNA polymerase α activity. O, No inhibitor; ●, 0.1 μ M-3'-NH₂-BV-dUTP; □, 0.2 μ M-3'-NH₂-BV-dUTP.

Fig. 5. Effect of 3'-NH₂-BV-dUTP on DNA polymerase β reaction in the presence of different concentrations of [³H]dTTP, with activated DNA as template

Other assay conditions were optimal for the DNA polymerase β activity. O, No inhibitor; □, 2 μ M-3'-NH₂-BV-dUTP; ●, 5 μ M-3'-NH₂-BV-dUTP.

To analyse the mechanism of inhibition of the DNA polymerase reaction by 3'-NH₂-BV-dUTP, we determined the extent of inhibition with increasing

Table 4. Kinetic parameters of inhibition of viral and cellular DNA polymerases by 3'-NH₂-BV-dUTP

DNA polymerase	K_m for dTTP (μ M)	K_i for 3'-NH ₂ -BV-dUTP (μ M)	K_m/K_i
HSV-1	0.6	0.13	4.6
α	4.7	0.1	47
β	11.1	1.3	8.5

concentrations of the substrate ([³H]dTTP). [³H]-dTTP was rate-limiting; the other three triphosphates were in excess. As demonstrated by Lineweaver & Burk (1934) plots, the inhibition was competitive with dTTP for all DNA polymerases studied [HSV-1 DNA polymerase (Fig. 3), DNA polymerases α (Fig. 4) and DNA polymerase β (Fig. 5)]. The K_m values for dTTP and K_i values for 3'-NH₂-BV-dUTP are presented in Table 4. The K_m/K_i ratio for HSV-1 DNA polymerase was 4.6, as compared with 8.5 for DNA polymerase β and 47 for DNA polymerase α . This indicates that 3'-NH₂-BV-dUTP inhibits the DNA polymerase α more specifically than it does the HSV-1 DNA polymerase. A similar conclusion was reached for BV-Ara-UTP, the 5'-triphosphate of BV-Ara-U (Descamps *et al.*, 1982). For BV-dUTP, however, the K_m/K_i ratio for HSV-1 DNA polymerase was about twice that for DNA polymerase α (Allaudeen *et al.*, 1981). Furthermore, the K_i of HSV-1 DNA polymerase for BV-dUTP was about 14 times lower than the K_i of DNA polymerase α (Allaudeen *et al.*, 1981); for 3'-NH₂-BV-dUTP it was two-fold higher (Table 4). Thus, unlike BV-dUTP, 3'-NH₂-BV-dUTP did not preferentially inhibit HSV-1 DNA polymerase.

Conclusion

3'-NH₂-BV-dUrd appears to be a potent and selective anti-herpes agent. It inhibits VZV and HSV-1 replication at a concentration of 0.03–0.1 μ g/ml, that is 2000–6000-fold lower than the concentration required to affect normal cell metabolism. Although the target for the antiviral action of 3'-NH₂-BV-dUrd may be identified as the DNA polymerase, the selectivity displayed by 3'-NH₂-BV-dUrd could not be attributed to a preferential inhibition of viral DNA polymerase. In fact, 3'-NH₂-BV-dUTP proved more inhibitory to cellular DNA polymerase α than to HSV-1 DNA polymerase. The selectivity in the anti-herpes activity of 3'-NH₂-BV-dUrd probably depends on a specific phosphorylation of the compound by the viral thymidine kinase, since 3'-NH₂-BV-dUrd has a much greater affinity (lower K_i) for HSV-1 thymidine kinase than for cellular thymidine kinase.

This specific phosphorylation would confine the action of 3'-NH₂-BV-dUrd to the virus-infected cell. Once converted to its 5'-triphosphate, 3'-NH₂-BV-dUrd would then interfere with both cellular and viral DNA synthesis, but, since 3'-NH₂-BV-dUrd is only processed to the 5'-triphosphate form by the virus-infected cell, its ultimate action at the DNA polymerase level would be restricted to the infected cell.

This investigation was supported by grant no. 3.0048.75 from the Belgian 'Fonds voor Geneeskundig Wetenschappelijk Onderzoek', grant no. 76/81-IV from the Belgian 'Geconcerteerde Onderzoeksacties', grant no. CH-47B from the American Cancer Society and grant no. CA-08341 from the U.S. Public Health Service. T. F. was supported by a fellowship from the 'Onderzoeksfonds, Katholieke Universiteit Leuven'. We are indebted to Dr. R. Busson, Dr. L. Colla and Professor H. Vanderhaeghe for the synthesis of 3'-NH₂-BV-dUrd, BV-Ara-U and BV-dUrd.

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