Antiviral Research, 15 (1991) 161–168

ADONIS 0166354291000295

AVR 00454

Short Communication

Carbovir: the (−) enantiomer is a potent and selective antiviral agent against human immunodeficiency virus in vitro

Jonathan A.V. Coates[1], Helen J. Inggall[1], Bridget A. Pearson[1], Charles R. Penn[1], Richard Storer[2], Christopher Williamson[2] and Janet M. Cameron[1]

[1]*Department of Virology and* [2]*Medicinal Chemistry II, Glaxo Group Research Limited, Greenford, U.K.*

(Received 18 May 1990; Accepted 21 September 1990)

Summary

In this paper we describe the in vitro antiviral activity of the (−) enantiomer of carbocyclic 2′,3′-deoxydidehydroguanosine, (−) carbovir, a nucleoside analogue that has selective and potent anti-HIV activity in a series of lymphocyte culture systems. The cellular cytotoxicity of this compound has also been evaluated in a number of systems and compared to the saturated dideoxynucleoside analogues AZT and ddC.

Carbovir; Nucleoside; HIV; AIDS

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS), which is characterised by a chronic suppression of immune functions and a concomitant increase in susceptibility to debilitating opportunistic infections (Gallo et al., 1983; Barré-Sinoussi et al., 1983; Fauchi et al., 1985).

In the search for selective anti-retroviral drugs much of the work has focussed on the HIV reverse transcriptase (RT) as the target. Some of the most promising antiviral agents have been 2′,3′-dideoxynucleoside analogues. 3′-Azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI) and

Correspondence to: J.A.V. Coates, Department of Virology, Glaxo Group Research Limited, Greenford Road, Greenford, Middlesex, UB6 0HE, U.K.

0166-3542/91/$03.50

2′,3′-dideoxyadenosine (ddA) are potent inhibitors of HIV replication in lymphocyte cultures, and protect the cells against virus-induced cytopathic effects at concentrations which are non-cytotoxic to the target cells in tissue culture (Mitsuya and Broder, 1986; Mitsuya et al., 1985). The apparent selectivity of these nucleoside analogues is probably due to specific inhibition of the viral RT by the nucleoside triphosphate. It has been shown that the 5′-triphosphate of AZT significantly inhibits the HIV RT at levels which have no measurable effect on host cell DNA polymerase alpha (Furman et al., 1986).

The same differential activity has also been shown for the triphosphate of ddT (Waqar et al., 1984).

A series of 2′,3′-dideoxynucleosides which incorporate a 2′–3′ double bond but which retain the natural base are also potent and selective inhibitors of HIV in lymphocyte cultures, e.g. 2′,3′-dideoxydidehydrothymidine (d4T), and 2′,3′-dideoxydidehydrocytidine (d4C), (Balzarini et al., 1986; Baba et al., 1987). Vince et al. (1988) described a series of carbocyclic nucleoside analogues, some of which exhibit potent antiviral activity against HIV in vitro. The most active of these is the carbocyclic analogue of 2′,3′-dideoxydidehydroguanosine [carbovir: (NSC 614846)].

In carbocyclic nucleosides, the oxygen atom of the furanose ring is replaced by a methylene group. They are therefore structurally analogous to natural and synthetic nucleosides, but since they lack the conventional glycoside linkage are stable to phosphorylases and hydrolases (Bennett et al., 1975).

The data reported by Vince et al. on carbovir were obtained for a racemic mixture of carbovir. We have now examined the (−) enantiomer of this nucleoside ana-

TABLE 1

Inhibition of HIV-induced cytopathic effects and cytotoxicity in different host cell lines

	Cell line					
	MT-4		C8166		JM	
	ID_{50}	CT_{50} (μg/ml)	ID_{50}	CT_{50} (μg/ml)	ID_{50}	CT_{50} (μg/ml)
(−) carbovir	0.31	100	0.12	>100	0.13	100
Racemic carbovir	0.52	100	-	-	-	-
ddC	0.001	100	0.004	>100	0.005	10
AZT	0.003	10	0.03	>100	>100	>100
ddT	0.5	>100	5.0	>100	100	>100

Antiviral (ID_{50}) and cytotoxicity (CT_{50}) values are in μg/ml. Evaluation of antiviral activity in MT-4 cells (Harada et al., 1985) was performed using the method of Pauwels et al. (1987, 1988) using HIV-1 strain RF (Popovic et al., 1984). The determination of antiviral activity in C8166 (Salahuddin et al., 1983) and JM cells (Schneider et al., 1977) was performed using the same method; C8166 cells were infected with HIV-1 strain RF and JM cells with HIV-1 strain GB8 (Dowsett et al., 1987). Compounds were tested for cytotoxicity at 100, 50, 10, 5 and 1 μg/ml and the CT_{50} was taken as the lowest concentration of compound tested that exhibited >50% inhibition of cell viability. (All viruses and cells used in this work were obtained through the MRC AIDS Directed Programme, U.K. AZT, ddT and ddC were obtained from Sigma Chemical Company, Dorset, U.K.).

logue for antiviral activity and toxicity in vitro.

The synthesis of (−) carbovir from the chiral carbocyclic nucleoside aristeromycin will be described in detail elsewhere (Williamson et al., in preparation). The antiviral activities and cytotoxicities of (−) carbovir, AZT, ddT and ddC in three different human CD4 lymphocyte cell lines in an MTT based microtitre assay (Pauwels et al., 1988), are summarised in Table 1.

In this assay, (−) carbovir exhibited a 50% inhibitory dose in MT-4, C8166 and JM cells of 0.31, 0.12 and 0.13 μg/ml, respectively. When compared for their inhibitory effects on the cytopathogenicity of HIV in MT-4, C8166 and JM cells, ddC proved to be the most potent in preventing cell death by the virus. In all three cell lines (−) carbovir was between 25 and 300-fold less active than ddC. In MT-4 and C8166 cells AZT was between 4 and 100-fold more active than (−) carbovir. AZT exhibited no measurable antiviral activity in HIV-infected JM cells, whereas (−) carbovir retained activity comparable to that seen in MT-4 and C8166 cells.

Similarly, ddT exhibited only weak activity in the JM cell line, while showing moderate activity in the C8166 and MT-4 cell lines. This suggests that JM cells are deficient in activating the thymidine but are capable of activating the cytidine class of pyrimidine nucleoside analogues. An analogous finding with AZT in MT-4 and ATH8 cells has been reported. Mitsuya et al. (1985), reported AZT to be fully protective at 1–5 μM in ATH8 cells, while Baba et al. (1987), showed the same level of activity in MT-4 cells treated with 0.02 μM AZT. These differences were also seen with ddT and are attributed to different phosphorylation patterns for thymidine analogues in the different cell lines.

When assayed for cytotoxicity in mock-infected cells, (−) carbovir reduced viability of MT-4 and JM cells by 50% at 100 μg/ml, while in C8166 cells no effect on cell viability was observed at 100 μg/ml, the highest concentration tested. AZT inhibited cell viability by 50% at 10 μg/ml in MT-4 cells, while ddC inhibited cell viability by 50% at 10 μg/ml in JM cells.

The inhibitory effects of (−) carbovir on HIV induced syncytium formation in C8166 and JM cells was examined (Table 2). In this assay (−) carbovir inhibited syncytium formation by 50% at 0.36 μg/ml in C8166 cells and at 0.1 μg/ml in JM cells. In comparison, ddC was 10 and 36-fold more active than (−) carbovir in JM and C8166 cells respectively. In C8166 cells (−) carbovir exhibited one third the activity of AZT. Again, AZT was inactive in JM cells, consistent with the results in the MTT assay.

The antiviral activities of (−) carbovir, ddC and AZT remain fairly constant, whether determined by the MTT or the inhibition of syncytium formation assay. Similarly, no significant differences in therapeutic index (ratio of antiviral ID_{50} to cytotoxicity CT_{50}) were apparent for the drugs tested in different cell lines or by different assays. This is, perhaps, not surprising. Nucleoside analogues which target the HIV RT, act early in the virus replicative cycle and ultimately inhibit viral cytopathic effect (cpe) and cell death. Compounds which act at a later stage of virus replication and/or which interact with cellular activities, may show greater differences in their potencies between cells and assays. The slight differences in antiviral activity seen for (−) carbovir in different cell lines are within experimental

TABLE 2

Inhibition of HIV-induced syncytium formation in two different host cell lines

	Cell line					
	C8166			JM		
	ID_{50}	CT_{50} (μg/ml)	TI	ID_{50}	CT_{50} (μg/ml)	TI
(−) carbovir	0.36	100	278	0.1	100	10^3
Racemic carbovir	0.85	100	118	0.3	100	333
ddC	0.01	>100	$>10^4$	0.01	10	10^3
AZT	0.1	>100	$>10^3$	>50	50	<1

TI: therapeutic index (ratio of CT_{50}/ID_{50}). Antiviral (ID_{50}) values and cytotoxicity values (CT_{50}) are in μg/ml. C8166 cells infected at an MOI of 0.001 infectious units/cell of HIV-1 strain RF (or strain GB8 with JM cells) were adsorbed for 1 h at room temperature. The cells were washed free of unadsorbed virus and aliquoted into 24-well plates containing serial dilutions of test compounds. After incubation at 37°C for 4–5 days in 5% CO_2/air, the cells were examined for HIV induced syncytia and these quantified. By reference to untreated infected controls the ID_{50} was calculated.

variation and show this compound to be equiactive in all the cell lines used. The same is true for the minor difference seen for ddC. (−) Carbovir was also evaluated for inhibition of viral p24 antigen release from HIV-infected C8166 and JM cells (Table 3). In these assays ddC was the most active compound, inhibiting p24 antigen synthesis by 98.9% and 99.7% at a concentration of 0.1 μg/ml in C8166 and JM cells respectively. Treatment of C8166 and JM cells with 0.1 μg/ml of (−) carbovir reduced p24 antigen synthesis by 67.1% and 44.2% respectively. AZT reduced p24 synthesis in infected C8166 cells treated with 0.1 μg/ml of drug by 98.2%. However, in line with its lack of antiviral activity in JM cells no reduction in p24 synthesis was observed at this concentration in this cell line.

The cytostatic effects of AZT, ddC and (−) carbovir were examined against a series of human CD4 lymphocyte cultures. When assayed by cell proliferation all three compounds showed some toxicity, but ddC was by far the most cytotoxic (Fig. 1). At a concentration of 10 μg/ml, (−) carbovir and AZT had little effect on cell proliferation for the first 2–3 days in U937 or CEM cells, but arrested proli-

TABLE 3

Inhibition of p24 internal core antigen synthesis

	Reduction at 0.1 μg/ml of drug (%)	
	C8166 cells	JM cells
(−) carbovir	67.1	44.2
ddC	98.9	99.7
AZT	98.2	<0.1
Virus control	<0.1	<0.1

Infections were performed in an identical manner to the inhibition of syncytium formation assays and incubated for 4 days at 37°C. Supernatant fluid from each well was decanted, cleared by centrifugation and assayed for p24 antigen using the Abbott ELISA kit. The procedures described by the manufacturers were followed exactly.

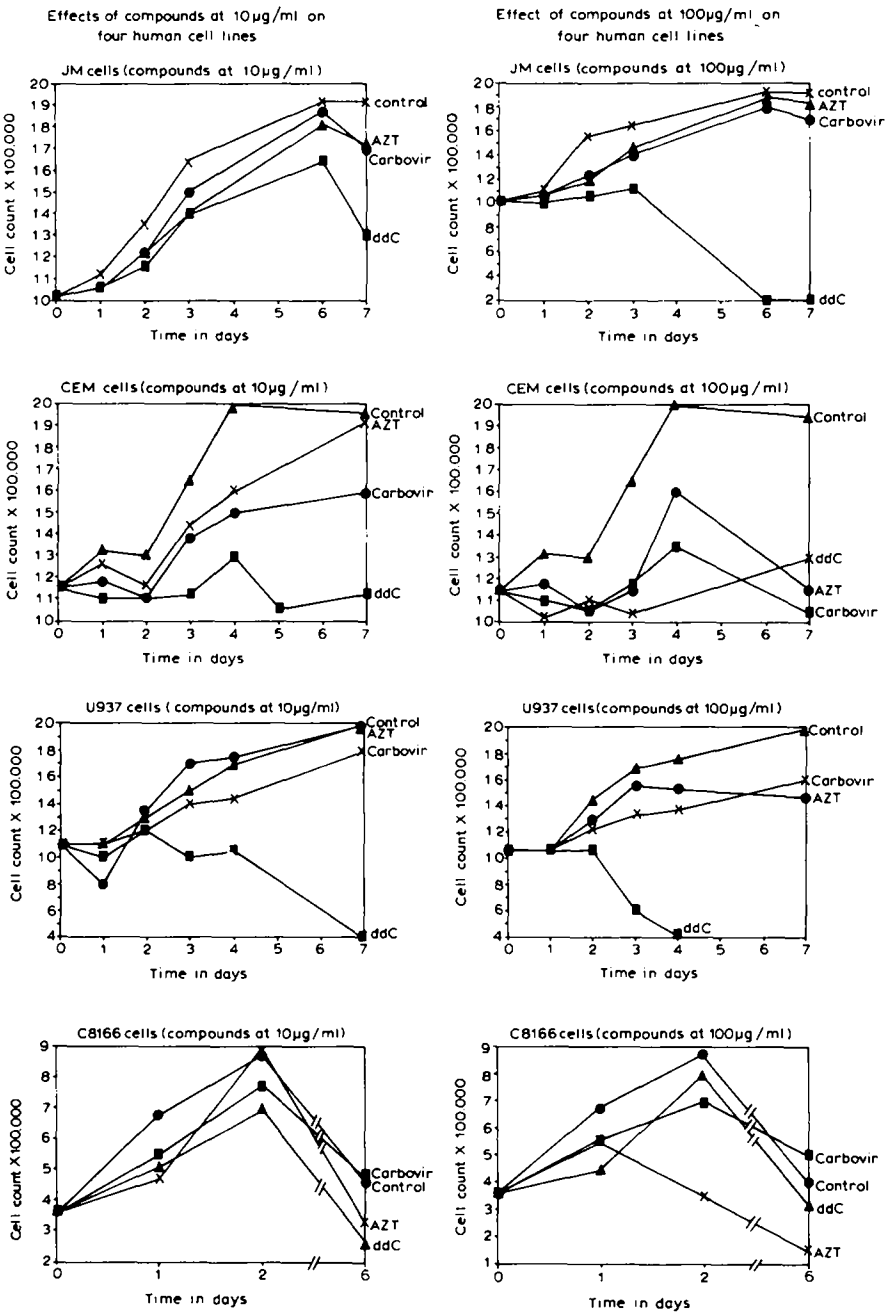

Fig. 1. Cytostatic and anti-metabolic assays. The cytostatic effects of the compounds were assessed by measuring inhibition of cell proliferation. Briefly, 75 cm^2 plastic tissue culture flasks were seeded with 10^5 cells/ml (3×10^4 in the case of C8166 cells) in the presence of either 100 µg/ml or 10 µg/ml of compound to a final volume of 15 ml. A sample was taken immediately and the flasks incubated at 37°C. This and subsequent samples were taken in triplicate and the cell number measured by counting in a haemocytometer.

TABLE 4

Inhibition of protein synthesis

Compound	Toxicity (ID_{50} μg/ml) in cell culture measured by incorporation of ^{14}C amino acids in		
	CEM cells	JM cells	U937 cells
(−) carbovir	5	20	65
AZT	15	20	65
ddC	<1	<1	2

Inhibition of protein synthesis in CEM, JM and U937 cells (Sundstrom and Nilsson, 1976) was determined by measuring inhibition of the incorporation of ^{14}C-protein hydrolysate into TCA-precipitate material. Suspensions of cells (approximately 10^6 cells/ml) in log-phase were washed once with growth medium. Test compounds were serially diluted in 96-well flat bottomed microtitre plates. 20 μl of cell suspension was added to each well and the plates incubated at 37°C. After 2 days incubation, 10 μl of growth medium containing 0.75 μCi of ^{14}C-protein hydrolysate was added to all wells and the plates incubated for a further three days at 37°C. After this time, lysis buffer was added to each well and the plates frozen and thawed twice. The contents of each well was then sampled in triplicate onto filter discs, washed in ice cold TCA, and assayed for TCA precipitate counts in a scintillation counter.

feration after that time. Neither AZT nor (−) carbovir at 10 μg/ml affected growth of JM cells for up to 7 days. At 100 μg/ml all three drugs had an inhibitory effect within two days in all four cell types. ddC at 10 μg/ml arrested growth of all four cell types within 2 days.

When measured by ^{14}C-amino acid incorporation into cells after 5 days, (−) carbovir was toxic at 5–65 μg/ml (ID_{50}), which is a similar range to that found for AZT (Table 4). ddC was more toxic.

There is therefore some evidence of toxicity of (−) carbovir for T-lymphocytes and monocytes from the above experiments, but the toxicity is no greater than that shown by AZT, and is less than that seen with ddC. The fact that (−) carbovir was cytostatic to human cell lines at concentrations more than 15 to 100-fold higher than the concentrations required to inhibit HIV replication, indicates this drug's selectivity as an anti-HIV agent. Although the therapeutic index of (−) carbovir is in general less than that of ddC or AZT, this does not lessen carbovir's potential as an antiviral agent, since the clinical toxicities, caused by AZT (anaemia) and ddC (peripheral neuropathy) would not be apparent from this type of analysis.

When examined for antiviral activity against a range of RNA viruses (−) carbovir did not demonstrate significant antiviral activity (ID_{50} > 100 μg/ml) against influenza A, human rhinoviruses type 14 and type 2, or respiratory syncytial virus, but was a potent inhibitor of visna maedi virus, feline and Friend leukaemia viruses (data not shown). This points to the selectivity of (−) carbovir as an anti-retroviral agent.

(−) Carbovir is approximately two-fold more active than the corresponding racemate. This suggests that most, if not all, of the antiviral activity of carbovir resides in the (−) enantiomer. Carter et al. (1990) have shown that the (+) enantiomer is at least 75-fold less active than the (−) enantiomer of carbovir. This is not surprising as the biological activities of nucleosides have been generally considered to reside in a single enantiomer, although Balzarini et al. (1990) have recently shown that

both enantiomers of carbocyclic bromovinyl deoxyuridine have antiviral activity.

In conclusion, the data indicate that (−) carbovir is a potent and selective inhibitor of HIV replication in vitro, with a high therapeutic index. As a representative of a carbocyclic series of nucleosides, (−) carbovir may also offer increased stability in vivo due to resistance to cleavage by phosphorylases and hydrolases. For these reasons, (−) carbovir may represent a potentially therapeutic drug for the treatment and control of HIV-induced disease (i.e., AIDS).

Acknowledgements

We wish to thank Ms J.M. Woods for performing the antiviral assays against influenza and respiratory syncytial viruses. We should also like to thank Drs A.J. Hay and A.S. Tyms for their help and encouragement at the MRC Collaborative Centre, Mill Hill, and the MRC AIDS Directed Programme for supplying reagents.

References

Baba, M., Pauwels, R., Herdewijn, P., De Clercq, E., Desmyter, J. and Vandeputte, M. (1987) Both 2′,3′-dideoxythymidine and its 2′,3′-unsaturated derivative (2′,3′-dideoxythymidine) are potent and selective inhibitors of Human Immunodeficiency Virus replication in vitro. Biochem. Biophys. Res. Commun. 142, 128–134.

Balzarini, J., Pauwels, R., Herdewijn, P., De Clercq, E., Cooney, D.A., Kang, G-J., Dalai, M., Johns, D.G. and Broder, S. (1986) Potent and selective anti-HTLVIII/LAV activity of 2′,3′-dideoxycytidinene, the 2′,3′-unsaturated derivative of 2′,3′-dideoxycytidine. Biochem. Biophys. Res. Commun. 140, 735–742.

Balzarini, J., De Clercq, E., Baumgartner, H., Bodenteich, M. and Greingl, H. (1990) Carbocyclic 5-iodo-2′-deoxyuridine (C-IDU) and carbocyclic (E)-5-(2-bromovinyl)-2′-deoxyuridine (C-BVdU) as unique examples of chiral molecules where the two enantiomeric forms are biologically active: interaction of the (+) and (−) enantiomers of C-IDU and C-BVdU with the thymidine kinase of herpes simplex virus type 1. Mol. Pharmacol. 37, 395–401.

Barré-Sinoussi, F., Chermann, J-C., Rey, R., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rosenbaum, W. and Montagnier, L. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220, 868–871.

Bennett, L.L., Shannon, W.M., Allan, P.W. and Arnett, G. (1975) Studies on the biochemical basis for the antiviral activities of some nucleoside analogues. Ann. N.Y. Acad. Sci. 255, 342–358.

Carter, S.G., Kessler, J.A. and Rankin, C.D. (1990) Activities of (−) carbovir and 3′-azido-3′-deoxythymidine against human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. 34, 1297–1300.

Dowsett, A.B., Roff, M.A., Greenaway, P.J., Elphick, E.R. and Farrar, G.H. (1987) Syncytia: a major site for the production of the human immunodeficiency virus? AIDS 1, 147-150.

Fauchi, A.S., Masur, H., Gelman, E.P., Markham, P.D., Hahn, B.H. and Lane, H.C. (1985) NIH Conference. The acquired immunodeficiency syndrome update. Ann. Intern. Med. 102, 800–813.

Furman, P.A., Fyfe, J.A., St Clair, M.H., Weinhold, K., Ridout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′ triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Gallo, R.C., Sarin, P.S., Gelman, E.P., Robert-Gurott, M., Richardson, E., Kalyanaraman, V.S., Mann, D., Sidhu, G.D., Stahl, R.E., Zolla-Pazner, S., Leibowich, J. and Popovic, M. (1983) Isolation of a

human T-cell leukaemia virus in acquired immune deficiency syndrome (AIDS). Science 220, 865–867.

Harada, S., Kayanagi, Y. and Yamamoto, N. (1985) Infection of HTLV-III/LAV in HTLV-1 carrying cells MT-2 and MT-4 and application in a plaque assay. Science 229, 563–566.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St Clair, M.H., Nusinoff-Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W. and Broder, S. (1985) 3′-Azido-3′-deoxythymidine (BW A509U) an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82, 7096–7100.

Mitsuya, I.T. and Broder, S. (1986) Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′-3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83, 1911–1915.

Pauwels, R., De Clercq, E., Desmyter, J., Balzarini, J., Gronbau, P., Herdewijn, P., Vanderhaeghe, H. and Vandeputte, M. (1987) Sensitive and rapid assays on MT4 cells for detection of antiviral compounds against the AIDS virus. J. Virol. Methods 16, 171–185.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Popovic, M., Samgadharan, M.G., Read, E. and Gallo, R.C. (1984) Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 297–300.

Salahuddin, S.Z., Markham, P.D., Wong-Staal, F., Franchini, G., Kalyanaraman, V.S. and Gallo, R.C. (1983) Restricted expression of human T-cell leukaemia-lymphoma virus (HTLV) in transformed human umbilical cord blood lymphocytes. Virology 129, 51–64.

Schneider, U., Schwenk, H.V. and Bornkamm, G. (1977) Characterisation of EBV-genome negative 'null' and 'T' cell lines derived from children with acute lymphoblastic leukaemia and leukaemic transformed non-Hodgkin lymphoma. J. Int. Cancer 19, 521–526.

Sundstrom, C. and Nilsson, K. (1976) Establishment and characterisation of a human histocytic lymphoma cell line (U937). Int. J. Cancer 17, 565–577.

Vince, R., Hua, M., Brownell, J., Daluge, S., Lee, F., Shannon, W., Lavelle, G., Qualls, J., Weislow, O., Kiser, R., Canonico, P., Schultz, R., Narayanan, V., Mayo, J., Shoemaker, R. and Boyd, M. (1988) Potent and selective activity of a new carbocyclic nucleoside analogue (carbovir: NSC 614846) against human immunodeficiency virus in vitro. Biochem. Biophys. Res. Commun. 156, 1046–1053.

Waqar, M., Evans, M., Manly, K., Hughes, R. and Huberman J. (1984) Effects of 2′,3′-dideoxynucleosides on mammalian cells and viruses. J. Cell Physiol. 121, 402–408.