ELSEVIER

Antiviral Research 27 (1995) 325–334

Antiviral Research

The ribonucleotide reductase inhibitor (*E*)-2′-fluoromethylene-2′-deoxycytidine (MDL 101,731): a potential topical therapy for herpes simplex virus infection

C. Gordon Bridges [a,*], S. Parvin Ahmed [a], Prasad S. Sunkara [b,1], James R. McCarthy [b,1], A. Stanley Tyms [a]

[a] *Marion Merrell Dow Research Institute Laboratories, MRC Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK*

[b] *Marion Merrell Dow Research Institute Laboratories, Cincinnati, OH, USA*

Received 24 November 1994; accepted 13 February 1995

Abstract

The ribonucleotide reductase inhibitor MDL 101,731 was examined for antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro and in combination with acyclovir in the murine zosteriform model of HSV-1 infection. The in vitro antiviral activity (IC_{50}) for both serotypes of HSV was similar and in the range 23–98 nM for Vero cells. Comparable activities were obtained against acyclovir-resistant viruses. In the zosteriform model, topical combination therapy of MDL 101,731 with acyclovir (5%:5% w/w) applied 48 h after infection was more effective than acyclovir alone and even appeared to promote lesion resolution.

Keywords: Ribonucleotide reductase; HSV; Zosteriform rash model; MDL 101,731

1. Introduction

Ribonucleotide reductases are responsible for the reduction of ribonucleotides (rNDPs) to deoxyribonucleotides (dNDPs) and are subject to allosteric control by nucleoside triphosphate (dNTP) concentrations. The herpes simplex virus (HSV) encoded ribonu-

* Corresponding author. Fax: +44 (81) 906 1395.

[1] Present address: Neurocrine Biosciences Inc., 3050 Science Park Road, San Diego, CA 92121, USA.

SSDI 0166-3542(95)00015-1

cleotide reductase (RR) is less sensitive to allosteric control than the cellular enzyme and consequently unrestricted synthesis of dNTPs takes place in infected cells – a potentially important contribution to viral DNA synthesis. The HSV RR is required for full expression of pathogenicity in mice or guinea pigs and therefore appears to represent a prime target for antivirals (Cameron et al., 1988; Brandt et al., 1991; Idowu et al., 1992). However, it is less clear whether the HSV RR is necessary for virulence in man because, although RR deficient mutants grow poorly in mouse cells in vitro, they can retain good growth characteristics in cells of another species (Goldstein and Weller, 1988; Jacobson et al., 1989).

Oral acyclovir (Zovirax) is the treatment of choice for herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections (reviewed by Reardon and Spector, 1991). Whilst an early study (Fiddian et al., 1983) suggested that topical treatment with 5% acyclovir (ACV) cream would be an effective and convenient therapy for orolabial infection, subsequent work removed this initial optimism (Shaw et al., 1985). The inefficiency of topical ACV treatment can be explained by its poor tissue penetration, but could also be a reflection of its inability to promote lesion resolution by reversing the pathological consequences of existing infection. The appearance of resistance to ACV in immunocompromised patients is an additional disadvantage which makes it a less attractive drug for use as a monotherapy.

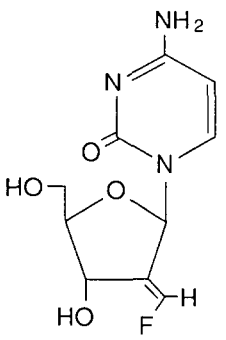
ACV is selectively phosphorylated in herpesvirus infected cells to acyclovir triphosphate (ACV-TP) which then competes with dGTP for binding to the target enzyme, herpesvirus-encoded DNA polymerase. In order to prevent the HSV RR-enhanced build-up of dGTP pools (which would compete with the dGTP analog ACV-TP) 2-acetylpyridine thiosemicarbazone inhibitors of RR have been tried in combination with ACV (Spector et al., 1985; Spector et al., 1989). Pools of dGTP were decreased as predicted, but unexpectedly there was also an increase in ACV-TP. This increase might be related to a perturbation of precursor pools, which, in infected cells, would normally compete with ACV for phosphorylation by thymidine kinase (Daikoku et al., 1991).

Recently, the potentiation of ACV by RR inhibition has been demonstrated in vivo and in vitro for wild-type and ACV-resistant viruses using 2-acetylpyridine 5-[(2-chloroanilino)thiocarbonyl]thiocarbonohydrazone, 348U87, a compound selected for reduced haematological toxicity (Spector et al., 1992). In this preliminary report, we examine the HSV antiviral activity of the RR inhibitor (*E*)-2′-fluoromethylene-2′-deoxycytidine (FMdC, MDL 101,731), an inhibitor of mammalian RR and antitumour agent (McCarthy et al., 1991; Sunkara et al., 1991). This compound (Fig. 1) was specifically designed for intracellular phosphorylation to the diphosphate form which irreversibly inhibits the enzyme.

2. Materials and methods

2.1. Viruses

Virus stocks were prepared from supernatant fluids of cells infected with HSV type 1 (strain SC16, strain 17i, strain S6, and strain TP2.5) or HSV type 2 (strain HG52). Stocks were stored in liquid nitrogen. Infectivity titres were determined by plaque assay.



(E) 2'-fluoromethylene-2'-deoxycytidine

Fig. 1. The structure of the ribonucleotide reductase inhibitor MDL 101,731 (FMdC).

2.2. Plaque-reduction assay

Inhibition of virus replication was measured by standard plaque-reduction assay (Tyms et al., 1981). Virus (~ 50 plaque forming units (PFU), 300-μl volumes in MEM) was adsorbed on confluent Vero or MRC-5 cell monolayers (1.78 cm^2) in 24-well plates for 1.5 h at room temperature, the inocula discarded, and triplicate monolayers overlaid with 0.5% agarose in media containing compounds at the indicated concentrations. The plates were then incubated at 36°C for 3 days after which they were fixed with 4% formalin-PBS overnight, the agarose plugs removed, and monolayers stained with methylene blue (0.3% w/v in PBS). Subsequently plaques were counted with the aid of a low power microscope. IC_{50} values were determined from line fit (95% confidence) of dose–response curves.

2.3. Animal model

The zosteriform rash model of HSV-1 infection as described by Simmons and Nash (1984) was used. Six- to 8-week-old female BALB/c mice (National Institute for Medical Research specific pathogen-free breeding colony) were shaved and depilated over the left flank. After 48 h, animals were infected by scarifying a 20 μl virus inoculum (HSV-1 strain SC16 5×10^4 PFU) in a dorsal position immediately lateral to the spine and in line with the spleen. The animals were coded and objective daily clinical scoring was performed according to the criteria of Lobe et al. (1991) with the addition of a score of 0.5 (infection only visible at inoculation site, no swelling) to describe a resolving lesion. Results were analyzed with the Kruskal–Wallis test with *P*-values adjusted for ties.

2.4. Drugs and drug delivery

For in vitro assays, drugs were diluted in medium from stock solutions of compounds prepared in water to a final concentration of 20 mM (10 mM for acyclovir), 0.2 μm

filtered, and stored at −20°C. For animal studies, ACV and RR inhibitor were formulated w/w in the paraffin-based E45 cream (Crookes Healthcare Ltd., Nottingham). Drugs were percutaneously applied to the inoculation site and dermatome twice a day starting 48 h postinfection (p.i.) and this regime was continued throughout the study. Each delivery contained approximately 3.75 mg drug (range 2.5–5 mg) for a 5% preparation. Untreated animals received E45 cream without additions. Elizabethan collars (IMS, Congleton, UK) were fitted to prevent ingestion during grooming.

2.5. Virus recovery

At 6 days post infection (d.p.i.) animals were culled and the brains removed, weighed and homogenized by repeated passage through a 21-gauge needle. Tissue was subsequently vortexed in DMEM and virus load measured by titration of 10-fold dilutions on Vero indicator cells for plaque assay as described above. Results were analyzed with the two-sample *t*-test.

3. Results

3.1. Antiviral effects of MDL 101,731 on acutely infected cells

The antiviral activity of MDL 101,731 was similar for both HSV-1 and HSV-2 with IC_{50} values in the range 23 to 98 nM (Table 1). When the antiviral activity of MDL 101,731 was compared with acyclovir, the ribonucleotide reductase inhibitor was, on average, 95 times more potent.

MDL 101,731 was also tested by plaque-reduction assay against ACV-resistant viruses, the thymidine kinase-deficient (TK^-) mutant (strain S6), a DNA polymerase mutant (strain TP2.5) of HSV type 1 strain SC16, the parental virus (Fig. 2). The IC_{50} for MDL 101,731 was similar for wild-type virus and acyclovir-resistant mutants ($\sim$ 10 nM). In contrast, the corresponding IC_{50} value for acyclovir against the TK^- strain was 50 μM and for the DNA polymerase mutant 80 nM, approximately 5000-fold and 8-fold less responsive, respectively, than MDL 101,731.

Table 1
In vitro antiviral effects (IC_{50} value ± S.D.) of MDL 101,731 or acyclovir in Vero cells

Virus	Expt.	MDL 101,731 (μM)	Acyclovir (μM)	Relative activity [a]
HSV-1 17i	1	0.023 ± 0.003	1.4 ± 0.12	×61
	2	0.023 ± 0.004	1.2 ± 0.04	×52
HSV-1 SC16	1	0.021 ± 0.003	2 ± 0.21	×95
	2	0.024 ± 0.003	1 ± 0.06	×45
HSV-2 HG52	1	0.098 ± 0.008	2.7 ± 0.10	×28
	2	0.021 ± 0.005	6.0 ± 0.18	×286

[a] Increased activity of MDL 101,731 over acyclovir assayed by plaque-reduction assay for 3 d.p.i.

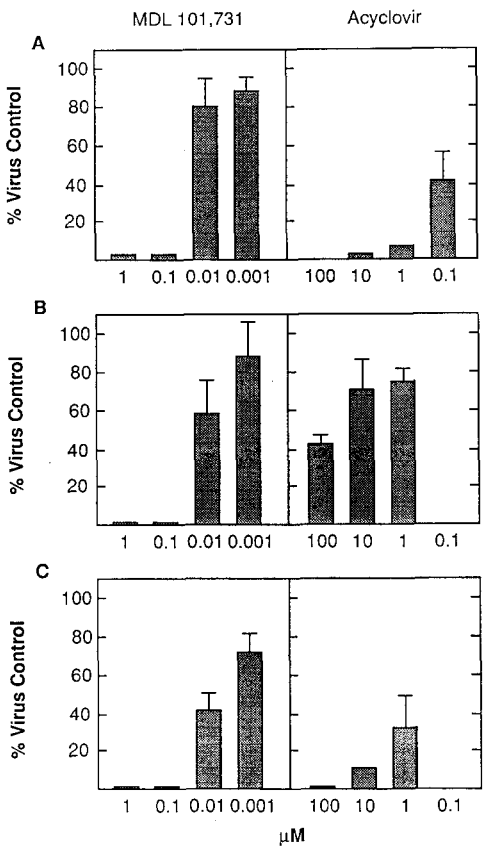


Fig. 2. The antiviral activity of MDL 101,731 or acyclovir against HSV-1 mutants. Plaque-reduction assay in MRC-5 cells. A: wild type (strain SC16). B: thymidine kinase-deficient mutant (strain S6). C: a DNA polymerase mutant (strain TP2.5).

With respect to the cytotoxicity of MDL 101,731, confluent monolayers of either Vero cells or human embryonic fibroblasts remained > 95% viable at 10 μM, as judged by Trypan blue dye exclusion, whereas the CC_{90} (measured by cell counts) for the cytostatic effect on proliferating cells was 1 μM (results not shown). Cells were able to grow at 100 nM MDL 101,731.

3.2. *Zosteriform model*

Clinical scoring in a representative experiment (Fig. 3) showed that topical combination therapy of 5% ACV with 5% MDL 101,731 was significantly better than ACV alone on days 4, 5 and 6 p.i. ($P = 0.016$, $P = 0.007$, $P = 0.005$, respectively). Combination therapy of 2.5% ACV with 5% MDL 101,731 was also significantly better than ACV alone on days 4 and 5 p.i. ($P = 0.031$, $P = 0.008$, respectively), but not on day 6 ($P = 0.452$). ACV alone, when compared to untreated controls, delayed, but did not

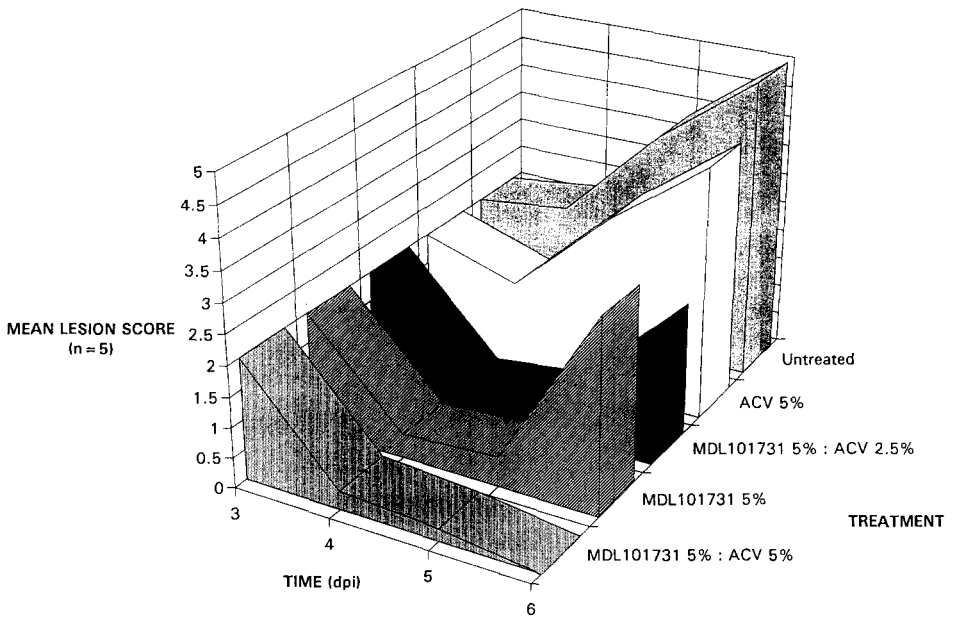


Fig. 3. The effect of treatment with MDL 101,731 alone or in combination with acyclovir on lesion score in mice infected with HSV-1 (strain SC16).

significantly improve, the mean lesion score on any day ($0.5 > P > 0.1$). Moreover, combination treatment applied at a late stage (48 h p.i.) appeared to reverse lesion pathology, whereas ACV alone merely delayed lesion progress. The effect of combination treatment with MDL 101,731 and ACV on lesion development is illustrated in Fig. 4, which shows that while the initial inoculation site is recognizable at 6 d.p.i. a confluent stage 5 lesion has not developed. In contrast, MDL 101,731 alone improved the lesion score on days 4 and 5 p.i. ($P = 0.011$, $P = 0.008$), but on day 6 there was an increase in lesion score ($P = 0.325$).

Virus was isolated from the brain in 4/5 untreated mice, 0/5 combination 5%:5% treated mice, 2/5 5%:2.5% treated mice, 1/5 5% MDL 101,731-treated mice and 0/5 5% ACV-treated mice (Fig. 5). All treatment groups had significantly less virus in the brain than untreated mice ($P < 0.05$). There was no significant difference in the virus load when any treatment group was compared with another treatment group ($P > 0.1$).

4. Discussion

Topical treatment of cutaneous HSV infection has the advantages of convenience and target site selection along with the prospect for maintenance of high local dose rates. However, in recurrent genital herpes, topical monotherapy with ACV is less effective at preventing virus shedding than oral therapy (Corey et al., 1983) and moreover has little effect on symptoms (Luby et al., 1984). On the other hand, while oral ACV treatment

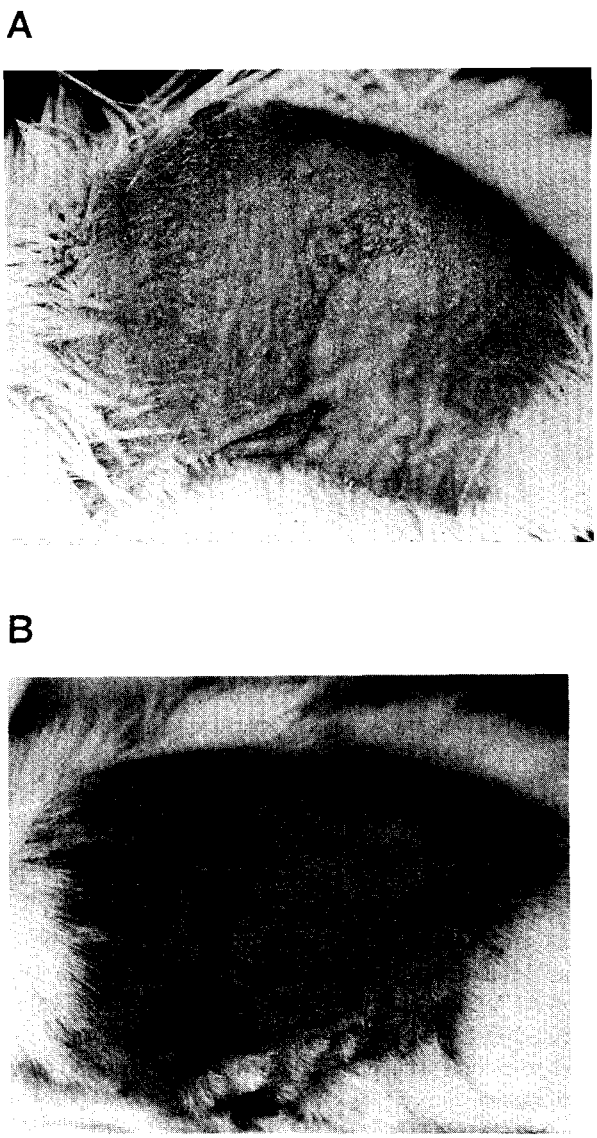


Fig. 4. The effect of topical combination treatment with MDL 101,731 and acyclovir (5%:5% w/w) on HSV-1 zosteriform lesion formation at 6 d.p.i., lesion score 0.5 (A). An untreated animal at the same time point is shown for comparison, lesion score 5 (B). There was dryness, with occasional induration of the skin in MDL 101,731-treated mice, but erythema was absent.

for herpes labialis or genital herpes shortens the duration of virus shedding, it has no influence on pain or lesion healing time (Nilsen et al., 1982; Raborn et al., 1987; reviewed by Whitley and Gnann, 1992). From this evidence it would appear that treatment for herpes infections would be improved if ACV were combined with a drug

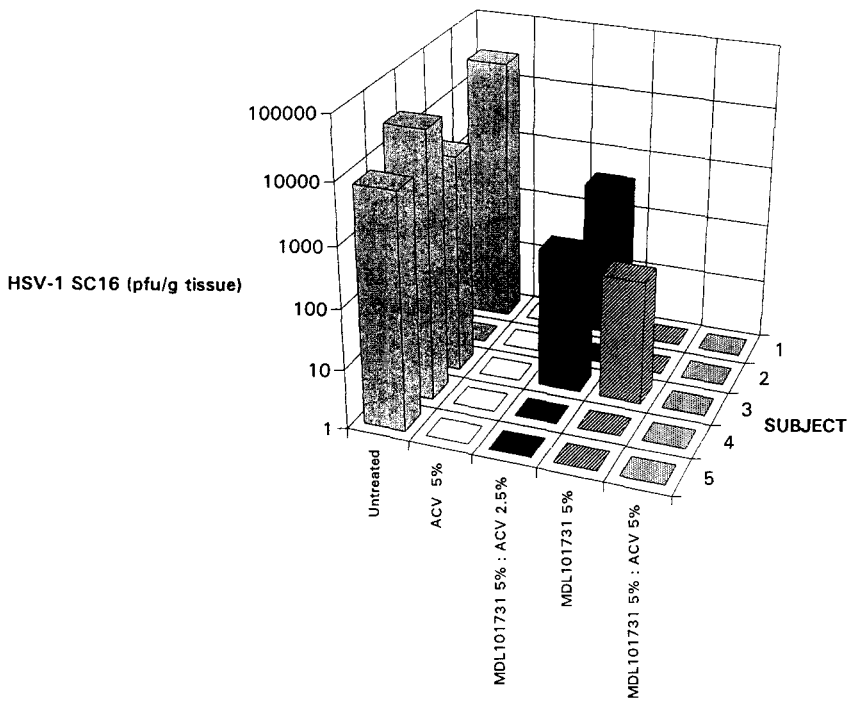


Fig. 5. Virus isolation from the brain of mice infected with HSV-1 (strain SC16) measured by plaque assay.

which could enhance its efficacy and reduce symptoms. Previously, combination treatment with a union of ACV and ribonucleotide reductase inhibitors has been proven highly effective in vitro and in vivo (Lobe et al., 1991; Spector et al., 1992). However, in these studies discrepancies were found between solo activity in vitro and topical monotherapy in the zosteriform rash model, indicating that ribonucleotide reductase inhibitors do not have independent activity in vivo. Moreover, in a recent human study, the combination of ACV and the ribonucleotide reductase inhibitor 348U87 was less effective than expected in the treatment of herpes labialis (Bernstein and Rheins, 1994). This was attributed to poor tissue penetration of the compound in the solar stimulator model used, but might also imply that viral RR activity can be dispensed with in human infections at sites where, due to cell division, deoxyribonucleotide pools are high (Goldstein and Weller, 1988).

We have shown here that MDL 101,731 has good activity in vitro against a range of HSV isolates, including ACV-resistant mutants, as well as activity when used as a topical monotherapy in mice. In addition, MDL 101,731 potentiated ACV for topical treatment of HSV-1 infection in mice. This was readily seen as a reversal in the state of existing rashes when applied as late as 48 h after infection. Conversely, and as expected, ACV monotherapy had little effect on lesions (Spector et al., 1992). The curtailment of lesions by MDL 101,731 is of particular relevance to management of herpes simplex virus infections since it implies that the associated pain and duration of sores may be

lessened by this type of compound. While it is unclear how MDL 101,731 reduces the severity of lesions, it is possible that it can influence the local inflammatory response to virus replication and, in turn, tissue damage. If this is the case then, in general, antivirals in combination with immunomodulators may prove beneficial for the topical treatment of herpes infections.

Topical monotherapy with ACV (5%) or MDL 101,731 (5%) influenced the number of mice succumbing to CNS spread of virus (1% MDL 101,731 monotherapy did not affect virus spread to the brain, data not shown). For ACV monotherapy the paradox of large lesion score together with low virus load in the brain is probably due to the systemic distribution of compound after establishment of the lesion. This apparent difference is likely to be accentuated by the late start of treatment. Combinations of ACV and MDL 101,731 were also effective in preventing spread of HSV infection, there being no evidence of antagonism between the two drugs. It is difficult to directly compare our findings with those of other workers due to the different treatment regimens employed but, taken together, the results presented here suggest that MDL 101,731 does have independent activity in the zosteriform rash model. However, MDL 101,731, when used in combination with ACV, offers the twin benefits of antiviral activity against acyclovir-resistant viruses and a sustained effect in the prevention of lesion formation.

With respect to both lesion severity and virus spread to the brain, the present data is insufficient to determine if this combination of ACV with MDL 101,731 is synergistic in vivo or merely additive. Moreover, the present results do not exclude the possibility that an optimal combination therapy is better than ACV alone with respect to prevention of virus spread. Further studies are underway to both define the interaction of MDL 101,731 and acyclovir, as well as the mechanism by which this RR inhibitor impedes zosteriform lesion formation.

Acknowledgements

We wish to thank the animal husbandry staff of NIMR for their care of the mice. Acyclovir was kindly provided by Dr. P. Collins, Wellcome Research Laboratories.

References

Bernstein, D.I. and Rheins, L.A. (1994) Solar stimulator-induced herpes labialis: use in evaluating treatment with acyclovir plus 348U87. Antiviral Res. 23, 225–233.

Brandt, C.R., Kintner, R.L., Pumfery, A.M., Visali, R.J. and Grau, D.R. (1991) The herpes simplex virus ribonucleotide reductase is required for ocular virulence. J. Gen. Virol. 72, 2043–2049.

Cameron, J.M., McDougall, I., Marsden, H.S., Preston, V.G., Ryan, D.M. and Subak-Sharpe, J.H. (1988) Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. J. Gen. Virol. 69, 2607–2612.

Corey, L., Benedetti, J., Critchlow, C. et al. (1983) Treatment of primary first episode genital herpes simplex virus infections with acyclovir: results of topical, intravenous and oral therapy. J. Antimicrob. Chemother. 12 (Suppl. B), 79–88.

Daikoku, T., Yamamoto, N., Maeno, K. and Nishiyama, Y. (1991) Role of viral ribonucleotide reductase in the increase of dTTP pool size in herpes simplex virus-infected Vero cells. J. Gen. Virol. 72, 1441–1444.

Fiddian, A.P., Yeo, J.M., Stubbings, R. and Dean, D. (1983) Successful treatment of herpes labialis with topical acyclovir. Br. Med. J. 286, 1699–1701.

Goldstein, D.J. and Weller, S.K. (1988) Herpes simplex virus type-1 induced ribonucleotide reductase activity is dispensible for virus growth and DNA synthesis: isolation and characterization of a ICP6 *lacZ* insertion mutant. J. Virol. 62, 196–205.

Idowu, A.D., Fraser-Smith, E.B., Poffenberger, K.L. and Herman, R.C. (1992) Deletion of herpes simplex virus type 1 ribonucleotide reductase gene alters virulence and latency in vivo. Antiviral Res. 17, 145–156.

Jacobson, J.G., Leib, D.A., Goldstein, D.J., Bogard, C.L., Schaffer, P.A., Weller, S.K. and Coen, D.M. (1989) A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. Virology 173, 276–283.

Lobe, D.C., Spector, T. and Ellis, M.N. (1991) Synergystic topical therapy by acyclovir and A1110U for herpes simplex virus induced zosteriform rash. Antiviral Res. 15, 87–100.

Luby, J.P., Gnann, J.W., Alexander, W.J. et al. (1984) A collaborative study of patient-initiated treatment of recurrent genital herpes with topical acyclovir or placebo. J. Infect. Dis. 150, 1–6.

McCarthy, J.R. and Sunkara, P.S. (1995) Design, synthesis and antitumour activity of an inhibitor of ribonucleotide reductase. In: D.B. Weiner and W.V. Williams (Eds), Chemical and Structural Approaches to Rational Drug Design, CRC Press, Boca Raton, pp. 3–34.

McCarthy, J.R., Matthews, D.P., Stemerick, D.M., Huber, E.W., Bey, P., Lippert, B.J., Snyder, R.D. and Sunkara, P.S. (1991) Stereospecific method to *E* and *Z* terminal fluoro olefins and its application to the synthesis of 2′-deoxy-2′-fluoromethylene nucleosides as potential inhibitors of ribonucleoside diphosphate reductase. J. Am. Chem. Soc. 113, 7439–7440.

Nilsen, A.E., Aasen, T., Halsos, A.M., et al. (1982) Efficacy of oral acyclovir in the treatment of initial and recurrent genital herpes. Lancet 2, 571–573.

Raborn, G.W., McGaw, W.T., Grace, M., Tyrrell, L.D. and Samuels, S.M. (1987) Oral acyclovir and herpes labialis: a randomised, double-blind, placebo-controlled study. J. Am. Dent. Assoc. 115, 38–42.

Reardon, J.E. and Spector, T. (1991) Acyclovir: mechanism of antiviral action and potentiation by ribonucleotide reductase inhibitors. Adv. Pharmacol. 22, 1–27.

Shaw, M., King, M., Best, J.M., Banatvala, J.E., Gibson, J.R. and Klaber, M.R. (1985) Failure of acyclovir cream in treatment of recurrent herpes labialis. Br. Med. J. 291, 7–9.

Simmons, A. and Nash, A.A. (1984) Zosteriform spread of herpes simplex virus as a model of recrudescence and its use to investigate the role of immune cells in prevention of recurrent disease. J. Virol. 52, 816–821.

Spector, T., Averett, D.R., Nelson, D.J., Lambe, C.U., Morrison, R.W., St Clair, M.H. and Furman, P.A. (1985) Potentiation of antiherpetic activity of acyclovir by ribonucleotide reductase inhibition. Proc. Natl. Acad. Sci. U.S.A. 82, 4254–4257.

Spector, T., Harrington, J.A., Morrison, R.W., Lambe, C.U., Nelson, D.J., Averett, D.R., Biron, K. and Furman, P.A. (1989) 2-Acetylpyridine 5-[(dimethylamino)thiocarbonyl]-thiocarbonohydrazone (A1110U), a potent inactivator of ribonucleotide reductases of herpes simplex and varicella-zoster viruses and a potentiator of acyclovir. Proc. Natl. Acad. Sci. U.S.A. 86, 1051–1055.

Spector, T., Lobe, D.C., Ellis, M.N., Blumenkopf, T.A. and Szczech, G.M. (1992) Inactivators of herpes simplex virus ribonucleotide reductase: haematological profiles and in vivo potentiation of the antiviral activity of acyclovir. Antimicrob. Agents Chemother. 36, 934–937.

Sunkara, P.S., Zwolshen, J.H., Lippert, B.J., Snyder, R.D., Matthews, D.P. and McCarthy, J.R. (1991) Antitumor activity of (*E*)-2′-fluoromethylene-2′-deoxycytidine (FMdC, MDL 101,731): a novel inhibitor of ribonucleotide reductase. Proc. Am. Assoc. Cancer Res. 32, 415.

Tyms, A.S., Scamans, E.M. and Naim, H.M. (1981) The in vitro activity of acyclovir and related compounds against cytomegalovirus infections. J. Antimicrob. Chemother. 8, 65–72.

Whitley, R.J. and Gnann, J.W. (1992) Acyclovir: a decade later. New Engl. J. Med. 327, 782–789.