2′-Deoxy-5-ethyl-β-4′-thiouridine inhibits replication of murine gammaherpesvirus and delays the onset of virus latency

A Barnes[1], H Dyson[1], NP Sunil-Chandra[1]†, P Collins[2] and AA Nash[1]*

[1]Department of Veterinary Pathology, University of Edinburgh, Summerhall, Edinburgh, UK
[2]GlaxoWellcome, Gunnels Wood Road, Stevenage, UK

*Corresponding author: Tel: +44 131 650 1000; Fax: +44 131 445 5770; E-mail: tony.nash@ed.ac.uk
†Present address: Department of Medical Microbiology, University of Kelaniya, Ragama, Sri Lanka

The antiviral thionucleoside analogue 2′-deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU) was shown to be a more potent inhibitor of gammaherpesvirus infection than acyclovir. This compound inhibits replication of murine herpesvirus (MHV)-68 in the lungs of mice when given 3 days post-infection. However, as with other nucleoside analogues, it was unable to prevent the establishment of latency, despite delaying the onset of latent infection in the spleen. In contrast, virus persistence in the lung was inhibited following drug treatment, although persistence was re-established in mice when treatment was suspended after 12 days. These data suggest that 4′-S-EtdU is a highly effective inhibitor of murine gammaherpesvirus replication and as such provides a powerful tool to study the pathogenesis of this virus *in vivo*.

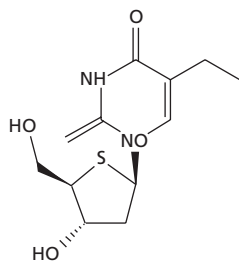Keywords: Thiopyrimidine; acyclovir; gammaherpesvirus; MHV-68

Introduction

Murine herpesvirus (MHV)-68 is a naturally occurring pathogen of small rodents (Blaskovic *et al.*, 1980) and has been designated a member of the *Gammaherpesviridae* on the basis of genome organization and sequence analysis (Efstathiou *et al.*, 1990a,b; Virgin *et al.*, 1996). MHV-68 can be propagated in epithelial or fibroblastoid cell lines, and persistently infects mouse-derived lymphoma cell lines of B cell origin (Sunil-Chandra *et al.*, 1993). Experimental infection of inbred mice, via the intranasal (i.n.) route, leads to an acute lung infection, lasting 10–12 days and a life-long latent infection of B lymphocytes (Sunil-Chandra *et al.*, 1992a,b). In the spleen, virus latency peaks around 15 days post-infection and is accompanied by a CD4 T lymphocyte driven splenomegaly (Ehtisham *et al.*, 1993; Usherwood *et al.*, 1996a). Long-term infection has been associated with the incidence of B cell lymphomas in elderly MHV-68-infected BALB/c mice (Sunil-Chandra *et al.*, 1994a; Usherwood *et al.*, 1996b). A number of similarities exist between MHV-68 and the human gammaherpesviruses Epstein–Barr virus (EBV) and Kaposi's sarcoma-related human herpesvirus 8 (HHV-8). This includes tropism for B lymphocytes, association with lymphoproliferative disorders and genetic homology, particularly with HHV-8. Consequently, MHV-68 is being used in potential models for these human virus infections.

The most effective anti-herpesvirus agents are presently considered to be the nucleoside analogues. A number of these have been used clinically to combat herpesvirus infections, such as acyclovir, penciclovir, famciclovir, valaciclovir and ganciclovir. Acyclovir is effective in combating acute and recurrent herpes simplex virus type 1 (HSV-1) and HSV-2 infections (Field & Darby, 1980) following activation by the thymidine kinase (TK) gene product. Acyclovir is less effective against the betaherpesviruses, such as human cytomegalovirus (HCMV), which lack a TK coding gene, and the gammaherpesviruses. MHV-68 encodes a functional TK with significant amino acid homology to the TK of other gammaherpesviruses (Pepper *et al.*, 1996). MHV-68 TK is functionally similar to that of EBV, since both are sensitive to acyclovir (Pepper *et al.*, 1996) and distinct from herpesvirus siamiri (HVS) and herpesvirus ateles, which despite having TK genes are not sensitive to acyclovir. However, both viruses are sensitive to other antiviral nucleoside analogues, such as 5-bromodeoxyuridine and 5-bromovinyldeoxyuridine (BVdU) (Sunil-Chandra *et al.*, 1993; Lin *et al.*, 1985; Honess *et al.*, 1989). Acyclovir has been used to treat individuals with acute or chronic EBV infection. However, as with other nucleoside analogues, this treatment did not affect the establishment or maintenance of viral latency, either *in vivo* (Yao *et al.*, 1989a; Luxton *et al.*, 1993) or *in vitro* (Lin *et al.*, 1985; Lin *et al.*,

Figure 1. The structural formula of 2′-deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU)

4′-S-EtdU is a pyrimidine nucleoside analogue with a sulphur atom replacing the 4-oxygen atom in deoxyribose.

1991). Furthermore, acyclovir is not effective in treating EBV-associated infectious mononucleosis (Yao *et al.*, 1989b).

2′-Deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU) is a cyclic thiopyrimidine analogue that is specifically activated, via phosphorylation of the 5′ hydroxyl group, by HSV-1 TK. It has a greater overall potency against alphaherpesviruses, such as HSV-1 and HSV-2 and varicella zoster virus (VZV) than BVdU, acyclovir or penciclovir (Rahim *et al.*, 1996).

In this paper we use the MHV-68 mouse model to study the effect of 4′-S-EtdU treatment on the control of the primary infection and on the establishment and maintenance of virus latency in the spleen and virus persistence in the lung. The efficacy of 4′-S-EtdU is compared with acyclovir, as this latter compound has been shown to be active against MHV-68 replication in fibroblast and lymphoid cell lines (Sunil-Chandra *et al.*, 1993), but only partially effective against MHV-68 infection in mice (Sunil-Chandra *et al.*, 1994b).

Materials and Methods

Mice
Female BALB/c mice were obtained from B and K Universal (Grimston, Aldbrough, Hull, UK) and infected i.n. at 3–4 weeks of age.

Virus
MHV-68 was obtained from the late Professor J Lesso (Institute of Virology, Academy of Science, Slovakia) (Blaskovic *et al.*, 1980). The virus stocks used were grown in BHK-21 cells from the sub-master stock of clone G2.4 (Efstathiou *et al.*, 1990a) and stored at −80°C.

Intranasal inoculation of mice
Lightly anaesthetized mice were inoculated i.n. with 4×10^5 p.f.u. of MHV-68 in a volume of 40 μl of PBS (as previously described by Sunil-Chandra *et al.*, 1992b).

Antiviral compounds
Acyclovir and 4′-S-EtdU (Figure 1) were obtained from GlaxoWellcome (Stevenage, UK). Both were solubilized by heating at 60°C for 45 min in distilled water at a concentration of 2 mg/ml. Stock solutions were filter-sterilized for use in tissue culture. 4′-S-EtdU stock solution was further diluted 1 in 6 with normal tap water for use as drinking water in the long-term antiviral treatment of mice. In some experiments short-term administration of antivirals (up to 12–13 days post-infection) was achieved by daily oral dosing (0.2 ml of acyclovir or 4′-S-EtdU in aqueous suspension equivalent to 40 mg/kg/mouse/day) by gavage.

Measurement of productive and latent virus infection
At each experimental time point the lungs and spleen were obtained from groups of 3–4 mice. Lungs were halved: one half was frozen at −80°C then homogenized and stored at −80°C, the other half was cut into a further eight pieces and co-cultivated for 5 days on BHK cells, under growth conditions as described for the infective centre assay (see below).

Virus titres were quantified by plaque assay, using BHK cells as the permissive cell line (as previously described by Sunil-Chandra *et al.*, 1992b). Cell monolayers were fixed and stained after 4 days incubation at 37°C and the infectious virus titre determined.

To determine the number of leukocytes that harbour latent virus in the spleen, an infective centre assay was used (Ehtisham *et al.*, 1993). Spleen cell preparations were lysed with water to remove red cells, serially diluted and then co-cultivated with 2×10^6 BHK cells, in 5 ml RPMI 1640 with 10% foetal calf serum for 5 days. The cell monolayers were fixed and stained and the number of infective centres quantified.
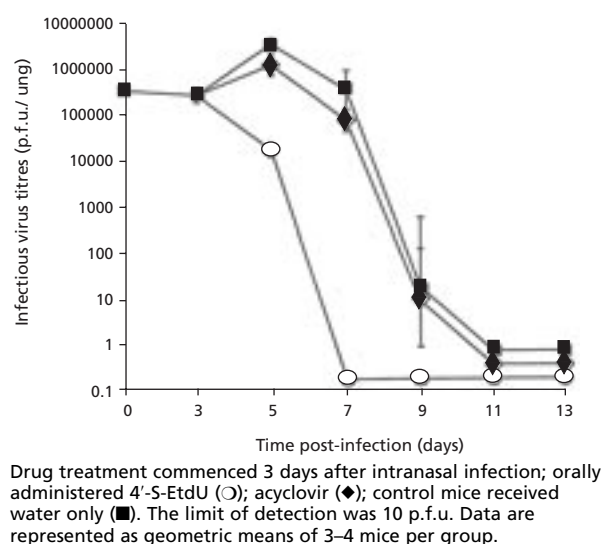
Sensitivity of virus to antiviral compounds using the plaque reduction assay
The sensitivity of MHV-68 stocks and infected tissue-derived virus to 4′-S-EtdU and acyclovir was determined by plaque reduction assay (50% effective concentration, EC_{50}), using the method described by Field & Darby (1980). Briefly, 200 p.f.u. of MHV-68 was added to 2×10^6 BHK cells in the presence of varying concentration of 4′-S-EtdU or acyclovir. The concentration of antiviral compound necessary to inhibit plaque formation by 50% (EC_{50}) was calculated by extrapolating the plaque inhibition as a percentage of the plaque count in the untreated controls.

Generation of virus isolates from latently infected tissues
Spleen leukocytes or small pieces of lung tissue were co-cultivated in Petri dishes with 2×10^6 BHK for 5 days, as

Figure 2. The effect of orally administered 4′-S-EtdU and acyclovir on the productive replication of virus in the lungs of BALB/c mice

Drug treatment commenced 3 days after intranasal infection; orally administered 4′-S-EtdU (○); acyclovir (◆); control mice received water only (■). The limit of detection was 10 p.f.u. Data are represented as geometric means of 3–4 mice per group.

described for the infective centre assay. The BHK cell monolayers were then removed with a sterile rubber scraper, homogenized and a plaque assay performed.

Statistics
Geometric means were used to evaluate average virus titres within groups of mice. This was because of the exponential nature of both the spleen infective centre titres and the productive virus titres in the lungs. Group means were compared using the two sample t-test with 95% confidence limits.
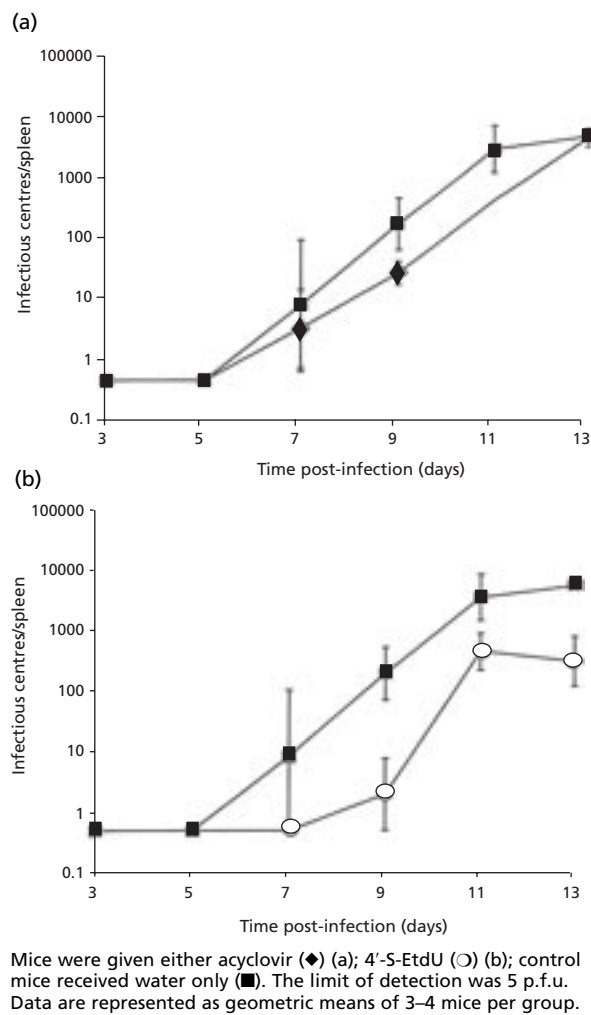
Results

Effect of 4′-S-EtdU on MHV-68 lytic replication in BHK cells
The EC_{50} of 4′-S-EtdU was evaluated in BHK cells by plaque reduction assay and found to be 0.035 µg/ml (0.128 µM). This compared with an EC_{50} of 0.4 µg/ml (1.78 µM) for acyclovir when performed under parallel test conditions. This indicates that 4′-S-EtdU is approximately 14 times more effective than acyclovir at inhibiting MHV-68 plaque formation.

4′-S-EtdU inhibits viral replication in the lungs of infected mice
A comparison was made of the effect of 4′-S-EtdU and acyclovir on the replication of MHV-68 in BALB/c mice following an intranasal infection with 4×10⁵ p.f.u. MHV-68. From day 3 post-infection and on subsequent days until the end of the experiment, groups of infected mice were

Figure 3. The establishment of latent virus infection in the spleen of mice given either acyclovir or 4′-S-EtdU 3 days after infection

Mice were given either acyclovir (◆) (a); 4′-S-EtdU (○) (b); control mice received water only (■). The limit of detection was 5 p.f.u. Data are represented as geometric means of 3–4 mice per group.
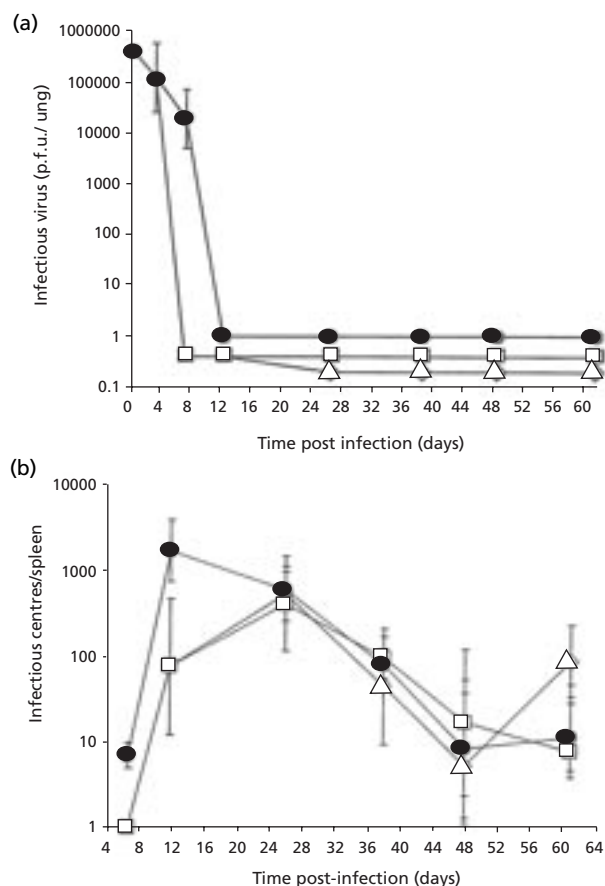
administered with either 4′-S-EtdU or acyclovir (40 mg/kg/day) in water. On days 3, 5, 7, 9, 11 and 13 a group of four mice were euthanized and the lungs and spleens removed for viral plaque assay and infectious centre assay (Figure 2). The 4′-S-EtdU treatment significantly reduced virus replication in the lung within 2 days of treatment (day 5 post-infection) and had eliminated infective virus from all mice within 4 days of treatment (day 7 post-infection). In contrast, acyclovir treatment did not appear to have any significant effect on virus replication in the lung during the period of the acute infection.

4′-S-EtdU was unable to prevent the establishment of splenic latency
The effect of 4′-S-EtdU on the establishment of virus latency was determined by an infective centre assay, using splenocytes taken from the infected mice described above.

Productive replication of virus in the lung (a) and latent infection of the spleen (b) in BALB/c mice. Continuous 4′-S-EtdU treatment from day 3 post-infection (□); withdrawal of 4′-S-EtdU after day 12 post-infection (△); control mice received water only (●). The limit of detection was 10 p.f.u. The compound was administered orally, under light anaesthesia, from day 3 to 12 post-infection (the control group was orally dosed with water) and then put onto 4′-S-EtdU supplemented drinking water. The data are represented as geometric means of 3–4 mice per group.

Groups of mice were administered either acyclovir or 4′-S-EtdU 3 days after infection with MHV-68. In the acyclovir-treated and the mock-treated control mice, infectious centres were first detected at day 7 post-infection and then increased exponentially up to 11 to 13 days post-infection (Figure 3a). In contrast, in mice receiving 4′-S-EtdU, infectious centres were first observed at day 9 post-infection and remained significantly lower than control or acyclovir treated mice at 13 days post-infection (with *P* values of <0.002, <0.02 and <0.05 for days 9, 11 and 13, respectively) (Figure 3b). The results showed clearly that whereas 4′-S-EtdU delayed the onset of latency in the spleen, neither acyclovir nor 4′-S-EtdU, given

from day 3 post-infection, were able to prevent the establishment of virus latency in lymphocytes.

Virus latency is maintained in the presence of 4′-S-EtdU

Since treatment with 4′-S-EtdU appeared to significantly delay the establishment of virus latency in the spleen, a second experiment was carried out to determine the effects of prolonged 4′-S-EtdU treatment. Mice were inoculated intranasally with $4×10^5$ p.f.u. MHV-68 and were orally dosed as before from day 3 post-infection with either 4′-S-EtdU (40 mg/kg/day) or a corresponding daily dose given in drinking water up to and including day 12 post-infection. After day 12 half the 4′-S-EtdU-treated mice had the drug treatment withdrawn, the other half were maintained on 4′-S-EtdU drinking water (0.33 mg/ml 4′-S-EtdU). This dose of compound delivered via drinking water was shown to be as effective as oral administration by gavage at inhibiting virus replication *in vivo* (data not shown). Mice were euthanized on days 3, 7, 12, 26, 38, 48 and 61 post-infection and lungs and spleens were removed for analysis as before. As with the previous experiment, 4′-S-EtdU treatment eliminated all detectable infectious virus from the lungs of infected mice within 4 days of treatment (Figure 4a). Continual 4′-S-EtdU treatment (from day 3 post-infection) or following 4′-S-EtdU withdrawal (from day 12 post-infection) did not affect the establishment or maintenance of virus latency in the spleen over the period of the experiment (Figure 4b).

The effect of 4′-S-EtdU on persistence of virus in the lung

After recovery from the acute infection, virus could still be detected in lung tissue using a variation of the infective centre assay (see Materials and Methods). To investigate the effect of 4′-S-EtdU on virus persistence, lung tissue was taken from mice at 38, 48 and 61 days post-infection and co-cultivated with BHK cells for 5 days. The presence of 'reactivated' or 'amplified' virus was determined by plaque assay. Virus was detected in all mice (9/9) infected with MHV-68 only. In contrast, only 1/12 mice had detectable virus following continuous treatment with 4′-S-EtdU. When 4′-S-EtdU treatment was withdrawn at 12 days post-infection, virus was again detected in the lungs of 10/12 mice.

Drug resistance testing of viral isolates

To determine whether 4′-S-EtdU resistant virus developed in infected animals during long-term treatment, virus isolates were obtained from spleens and lungs of mice sampled on days 38, 48 and 61. The sensitivities of isolates derived from the 4′-S-EtdU-treated groups were compared to both the untreated group and the original virus stocks, using an ED_{50} assay. None of the virus strains isolated from the

infected groups, whether 4′-S-EtdU treated or not, was resistant to the compound *in vitro*.

Discussion

In this study we show that 4′-S-EtdU is a potent inhibitor of MHV-68 replication *in vitro* and *in vivo*. 4′-S-EtdU was over 10 times more efficient than acyclovir at inhibiting MHV-68 plaque formation on BHK cells. Acyclovir has previously been reported to be a poor inhibitor of productive viral replication in the lungs of mice during acute MHV-68 infection (Sunil-Chandra *et al.*, 1994b) and in preventing a lethal MHV-68 infection in SCID mice (Neyts & De Clercq, 1998). In the present study, using acyclovir at 40 mg/kg mouse (daily oral dosing) had no significant effect on the infectious virus levels of the mouse lungs during the acute infection. In contrast, 4′-S-EtdU, when given at the same dose, dramatically reduced the infectious viral load in the lungs within 2 days of treatment and had eliminated all detectable infectious virus by day 4 post-treatment.

Acyclovir treatment of infected mice did not appear to affect the establishment of virus latency in the spleen as determined by the infective centre assay. In contrast, 4′-S-EtdU treatment delayed the appearance of infective centres in the spleen by up to 2 days and the number of infective centres remained significantly lower than the untreated controls, up to 13 days post-infection. This result strongly suggests that latent infection of B lymphocytes is established prior to the appearance of infectious centres in the spleen and that starting treatment 3 days post-infection was too late to influence the pattern of latent infection. The fact that treatment did have an effect on the initial latent viral load in lymphocytes implies that the severity of the acute infection does directly affect the initial levels of virus latency established. Consequently, if drug treatment is started earlier than day 3 post-infection, then this might well influence the establishment of viral latency. Experiments to test this hypothesis are in progress.

Although long-term treatment with 4′-S-EtdU delayed the establishment of virus latency in the short term, a latent virus infection was eventually established. Similar observations have been made with other antiviral nucleoside analogues in the treatment of herpesvirus infections, both *in vitro* and *in vivo* (Yao *et al.*, 1989a; Lin *et al.*, 1985, 1991; Luxton *et al.*, 1993; Sunil-Chandra *et al.*, 1993, 1994). For example, acyclovir is a potent inhibitor of the replication of HSV, however it does not prevent the establishment of a latent infection in neurons. Similarly, patients with EBV-associated infectious mononucleosis showed little or no direct therapeutic benefit from acyclovir, even though this compound controlled a chronic infection in the nasopharynx of EBV patients (Luxton *et al.*, 1993). Despite these findings, the rapid elimination of productively replicating virus during the acute infection may still be of therapeutic benefit. Evidence from betaherpesviruses, such as HCMV, indicates the sites and severity of recurrent disease appear to be linked to the sites and severity of the primary infection (Balthesen *et al.*, 1993).

The ability of 4′-S-EtdU to eliminate persistent virus from the lungs of long-term infected mice is extremely interesting in light of the recent information on the nature of MHV-68 persistence. Stewart *et al.* (1998) have shown that the virus genome exists predominantly in a circular form, indicative of a latent infection, although linear genome is also present, indicating a productive infection. Furthermore, alveolar epithelial cells were observed to express viral tRNA, a known molecular marker of the latent infection (Bowden *et al.*, 1997). In the light of this information it would appear that 4′-S-EtdU acts by blocking productive virus infection in the lung, but on withdrawal of the antiviral compound, infection can be re-established either from latent sites in the lung or via latently infected B cells (Stewart *et al.*, 1998). Infections and diseases of the respiratory tract are observed with other gammaherpesvirus infections, notably those associated with EBV. This includes diseases such as undifferentiated nasopharyngeal carcinoma (de The, 1982), oral hairy leukoplakia (Thomas *et al.*, 1991) and cryptogenic fibrosing alveolitis (Egan *et al.*, 1995).

In conclusion, 4′-S-EtdU is an excellent inhibitor of the productive replication of MHV-68, and as such is a very useful reagent for studying viral pathogenesis and latency, in the absence of productive viral replication, both *in vivo* and *in vitro*. The ability to interrupt virus replication at any time during the primary infection opens up the prospect of establishing latent infections in transgenic mice deficient in genes involved in antiviral immunity, which would otherwise undergo a fatal virus disease. This compound can also provide valuable information about early events in gamma-herpesvirus infection, in particular, in understanding the progression towards virus latency.

Acknowledgements

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