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STUDIES ON THE ANTIVIRAL ACTIVITY OF 5′-AMINO-2′,5′-DIDEOXY-5-IODOURIDINE (AIU) AGAINST HERPES VIRUSES IN VIVO AND IN VITRO

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5′-Amino-2′,5′-dideoxy-5-iodouridine (AIU) is known to have antiviral activity against herpes simplex virus type 1 (HSV-1) in cell cultures but is less potent than the parent compound 5-iodo-2′-deoxyuridine (IDU). Studies on its activity in vivo are limited. AIU showed antiviral effects in BHK cells against wild-type HSV-1 but not a thymidine kinase-negative (TK^-) mutant, indicating the importance of phosphorylation of the compound by HSV-1-induced thymidine kinase for antiviral effects. When cells were coinfected with the TK^- mutant and a wild-type TK^+ strain, both strains were inhibited by AIU, suggesting that the failure to phosphorylate AIU accounts for the resistance of the TK^- strain alone. AIU failed to limit death after systemic HSV-1 infection of mice when the drug was administered parenterally or orally, although IDU in similar treatment regimens was effective. Tested for efficacy in a local HSV-1 skin infection of mice, topical AIU in a petrolatum ointment or dimethyl sulphoxide (DMSO) did not reduce titres of virus in the skin or modify the clinical course of infection, whereas topical application of IDU in DMSO caused a significant reduction in the titres of virus in the skin and reduced both the occurrence and the severity of lesions. When administered subcutaneously or orally AIU had a slight antiviral effect against HSV-1 infection in the skin of mice. Moreover, intraperitoneally (i.p.) administered AIU limited HSV-1 replication in peritoneal cells of i.p. infected mice, indicating that AIU is inherently antiviral in vivo. The poor antiviral activity of AIU in vivo compared with IDU is attributed to its lower antiviral potency as judged by its activity in cell cultures and its inability to enter neural tissue.

AIU (5′-amino-2′,5′-dideoxy-5-iodouridine) antiviral herpes simplex virus

INTRODUCTION

Many pyrimidine nucleosides have been described as inhibitors of herpes virus replication, though some, e.g. 5-iodo-deoxyuridine (IDU), have the disadvantage of being incorporated into the DNA of normal, rapidly proliferating cells and consequently have

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proved to be toxic and in some cases mutagenic or carcinogenic [16, 17, 24, 25]. Recently, pyrimidine deoxyribonucleosides have been described which have antiviral activity but little or no toxicity to normal cells. Examples of such compounds are 5′-amino-2′,5′-dideoxy-5-iodouridine (AIU) [5], *E*-5-(2-bromovinyl)-2′-deoxyuridine [7], 5-methoxymethyl-2′-deoxyuridine [2], 5-propyl-2′-deoxyuridine [9], 5-propenyl-2′-deoxyuridine [6], and 5′-methylthiomethyl-2′-deoxyuridine [8]. The selectivity of these compounds appears to rest in part in their specific phosphorylation by herpes virus-induced thymidine kinase in the absence of phosphorylation, at least to any appreciable extent, in uninfected cells, as has been demonstrated for AIU [3] and the halogenovinyl-deoxyuridines [11].

Whilst AIU has been shown to be effective against herpes simplex virus type 1 in cell culture systems [5], its in vivo efficacy, which depends on many factors of which antiviral potency is just one, has not been extensively investigated. AIU has been reported to be effective in the treatment of experimental herpes keratitis in the rabbit [1, 28] when applied topically. However, high concentrations of AIU were required, either in solution or as an ointment, to achieve a therapeutic effect, and others [19] have been unable to repeat these results. We have studied the efficacy of systemically administered AIU against lethal systemic HSV-1 infections of mice and also antiviral activity against localised herpetic infections of mice. We present results which show that AIU inhibits the replication of HSV-1 in vivo, but suggest that the drug may not be useful in the treatment of clinical disease.

MATERIALS AND METHODS

Viruses

A wild-type strain of HSV-1, S3, of limited passage in cell culture, the laboratory strain of HSV-1, HFEM, and the thymidine kinase-negative (TK^-) mutant (B2006) of HSV-1 [12] were obtained from Dr. G.R.B. Skinner, Department of Medical Microbiology, University of Birmingham, U.K. Stocks were grown in BHK cells [33] and stored at -70°C.

Chemicals

5-Iodo-2′-deoxyuridine was purchased from International Enzymes Ltd., Windsor, U.K. AIU was synthesised as previously described [22] by the Chemical Development Department, G.D. Searle. Dimethyl sulphoxide (DMSO) and carboxymethyl cellulose were purchased from Sigma Chemical Co.

Animals

Female C3H/He mice were bred in our own SPF breeding unit. Female Webster Schneider (WS) mice were obtained from A. Tuck and Son Ltd., Battlesbridge, Essex, U.K. Mice were used between 6 and 10 weeks of age when they weighed between 18 and 25 g and were maintained at 22°C with unlimited access to water and a diet of RHM PMD cubes, Labsure Ltd., Poole, U.K.

Tissue culture assays

The antiviral activity of compounds was determined by a virus yield reduction assay. Confluent monolayers (25 cm^2) of BHK cells were cultured in Eagle's minimal essential medium, Dulbecco's modification, containing 10% calf serum (Flow Laboratories, Ltd.) and were infected with virus, 10 p.f.u./cell, and incubated for 1 h at 37°C with gentle agitation. The residual inoculum was removed, the monolayers washed twice with medium and fresh medium (5 ml) added with drug at the required concentration. After 24 h culture at 37°C, cells were scraped off the flasks into the medium, disrupted by ultrasonic vibration (Mettler Electronics ultrasonic cleaning bath) and frozen to -20°C. Virus yields were determined by a suspension plaque assay [31].

In vivo studies

C3H/He mice infected intraperitoneally (i.p.) with 10–100 × LD_{50} HSV-1 died of encephalitis, usually within 5–12 days. Compounds were given by the indicated route 8 h post-infection and twice daily (approximately 9 a.m. and 5 p.m.) for 5 days thereafter. The survival time (t) of mice in hours was obtained from records prepared twice daily. Records were made for 21 days from the day of infection, although no further deaths occurred after 16 days post-infection in any of the studies reported here. Significant differences in the survival times of different groups of mice were tested for by calculating χ^2 values by the logrank method [26]. To follow the course of virus replication in the peritoneum after infection and drug treatment, mice were sacrificed at the prescribed time and injected i.p. with 5 ml ice-cold tissue culture medium. The abdomen was gently massaged and the culture medium recovered from the peritoneal cavity. The harvested fluid was subjected to ultrasonic vibration to release virus from the cells present and the total extract was assayed for virus.

Localised herpes virus skin infection of mice was carried out essentially as described elsewhere [29]. WS mice were depilated and infected with HSV-1 by intradermal inoculation at a single site on the exposed flank with 0.05 ml HSV-1 suspension, 10^6 p.f.u./0.05 ml. Mice were treated with compound by the indicated route 4 h post-infection and twice daily (approximately 9 a.m. and 5 p.m.) for 4 days thereafter. Animals were scored daily for up to 10 days for the incidence of lesions and were graded for severity: 0, no lesion; 1, single lesion at site of inoculation; 2, multiple discrete lesions; 3, multiple lesions fused to form a zosteriform band from dorsum to ventrum. Mice were individually identified and the time (d) in days from infection until the first lesion appeared was recorded for each animal. Significant differences in the mean values of d of different groups of mice were tested for by the Student's t-test. Mortality in infected control mice was rarely more than 50% and lesions in surviving mice usually healed within 14 days. The titre of virus in the skin 5 days post-infection was determined by removing a 1 cm diameter circle of tissue, centred on the site of inoculation, from each of several mice. Samples were pooled, three mice per group, finely minced with scissors and a 10% w/v homogenate in tissue culture medium prepared using a Teflon/glass homogeniser. Homogenates were further disrupted by ultrasonic vibration (5 min), centrifuged (3000 × g, 5

min) and the supernatants stored at -20°C until assayed. Significant differences in the geometric mean virus titres in skin homogenates from different groups of mice were tested for by the Student's *t* test.

RESULTS

Antiviral activity of AIU in cell culture

In BHK cell cultures infected with HSV-1, AIU was less effective than IDU in reducing the yield of progeny virus measured 24 h post-infection. IDU reduced the yield of virus by $\log_{10}$ 2.0 at 6 μg/ml, whilst a concentration of 36 μg/ml AIU was required to achieve a comparable reduction in virus yield (Fig. 1). The requirement for HSV-1-induced thymidine kinase to be present in the infected cell in order that AIU show antiviral activity is indicated by the high resistance of the TK^- mutant of HSV-1 to AIU (Fig. 1). In order to determine whether the TK^- mutant was inherently susceptible to AIU after initial phosphorylation of the nucleoside, BHK cells were doubly infected with a syncytial plaque-forming wild-type strain of HSV-1 (HFEM) and with the non-syncytial plaque-forming TK^- mutant. Cell cultures were infected at 10 p.f.u./cell with either HFEM, B2006 or a mixture of HFEM and B2006 both at 10 p.f.u./cell. Infected cultures were incubated for 24 h with AIU (50 μg/ml) or without drug and the virus yields determined. The use of virus isolates with different plaque morphologies permitted us to determine the yield of virus of each type from the mixed infection. HFEM showed the expected susceptibility of wild-type HSV-1 to AIU and the virus yield was reduced by $\log_{10}$ 3.3. B2006 alone was resistant to AIU. In the mixed infected cultures the yield of both HFEM and B2006 were reduced by ca. $\log_{10}$ 3 in the presence of AIU (Table 1).

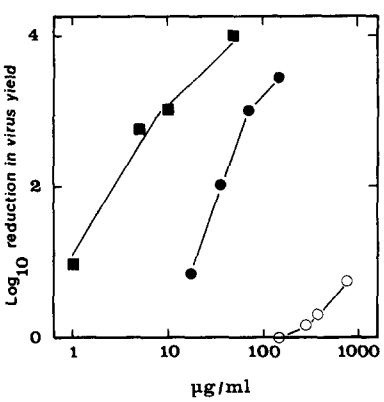

Fig. 1. Effect of AIU and IDU on the growth of HSV-1 in BHK cells. Cultures were infected with wild-type HSV-1 and treated with IDU (■) or AIU (●), or infected with TK^- HSV-1 and treated with AIU (o), and incubated at 37°C. Cultures were harvested after 24 h, lysed and subsequently assayed for infectious virus. Yields of virus ($\log_{10}$ p.f.u.) in drug-treated cultures were compared with yields from untreated controls.

TABLE 1

Effect of AIU on the yield of wild-type (HFEM) and TK$^-$ (B2006) HSV-1 following single or mixed infection of BHK cells

Virus	Virus yield (p.f.u./10^7 cells after 24 h)	
	- AIU	+ AIU[a]
HFEM	2.0×10^8 [b]	6.5×10^4
	1.0×10^8	9.5×10^4
B2006	3.5×10^8	3.0×10^8
	4.9×10^8	3.7×10^8
HFEM + B2006	7.5×10^7	6.5×10^4
	9.0×10^7	4.5×10^4
	1.5×10^8	7.0×10^4
	1.9×10^8	2.4×10^5

[a] AIU, 50 μg/ml.
[b] Results from replicate cultures.

AIU treatment of systemically infected mice

C3H mice were infected i.p. with HSV-1 (S3; 10^5 p.f.u./mouse) and treated i.p. with a solution of AIU, 1000 mg/kg/dose commencing 8 h post-infection. Such treatments over 5 days (see Materials and Methods) failed to afford significant protection and at higher doses of AIU drug toxicity was observed. Other treatment procedures involving more frequent i.p. administrations of AIU in solution also failed to protect mice. Lethally infected C3H mice were also treated by the oral route with AIU as a suspension in 0.5% carboxymethyl cellulose. Administered by gavage at 250 mg/kg commencing 8 h post-infection, AIU failed to protect mice from infection ($P>0.05$), whilst IDU administered in a similar regimen afforded significant protection ($P<0.001$) (Fig. 2).

The failure of AIU to exhibit significant antiviral activity in vivo may have been due to factors unrelated to its antiviral potency such as rapid metabolism or poor bioavailability. We therefore attempted to assess the antiviral activity of AIU in a more localised environment in vivo – the peritoneal cavity – where drug metabolism and bioavailability would bear less on the result. C3H mice were infected with HSV-1 (S3; 10^7 p.f.u./mouse i.p.) and divided into two groups. One group was treated with AIU, 250 mg/kg i.p. in solution 2 h and 8 h post-infection; the second group served as a control. Three mice from each group were sacrificed at 2, 8, 24 and 48 h post-infection; the peritoneal cells of each mouse were harvested into 5 ml of tissue culture medium and lysed by ultrasonic vibration. The lysates were assayed for virus and the mean virus titres are shown in Fig. 3. The peritoneal cells of control mice supported the replication of virus. Peak titres of

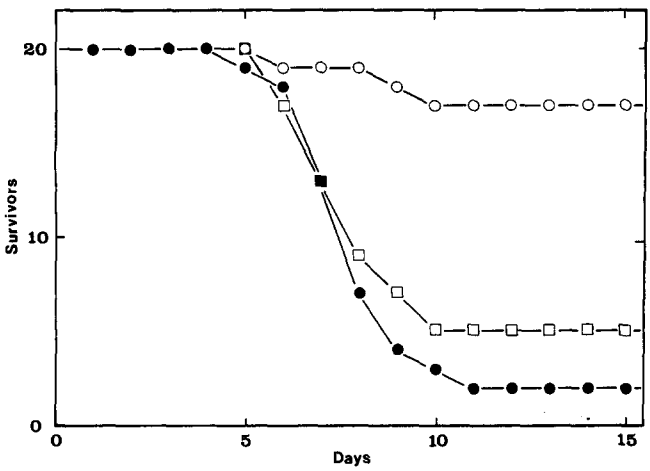

Fig. 2. Comparison of the effects of IDU (o) and AIU (□) against HSV-1 infection of C3H/He mice. Treatments by the oral route at 250 mg/kg 8 h post-infection and twice daily for 5 days thereafter. •, Infected control.

$10^{5.4}$ p.f.u./ml HSV-1 were obtained in lysates of cells harvested from mice 24 h post-infection and titres of $10^{4.5}$ p.f.u./ml were recovered from mice sacrificed 48 h post-infection. However, in AIU-treated mice the virus failed to replicate to the same extent. Virus, $10^{3.5}$ p.f.u./ml, was recovered from lysates of cells obtained from mice 24 h post-infection, but no virus ($<10^2$ p.f.u./ml) was detected in cells harvested from mice 48 h post-infection.

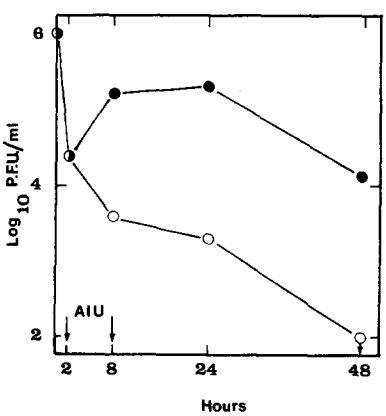

Fig. 3. Effect of AIU on the growth of HSV-1 in mouse peritoneum. Mice were infected i.p. with HSV-1 and treated with AIU (o) i.p. 2 and 8 h post-infection. Groups of mice were sacrificed at the indicated times, the peritoneal contents harvested, lysed and assayed for virus. •, Infected control.

AIU treatment of HSV-1 skin infection of mice

It was considered possible that AIU failed to protect systemically infected mice, because sequelae leading to death results from virus replication in a privileged site, e.g. neural tissue, inaccessible to the drug. Therefore, a second animal model was examined in which mice were infected intradermally with HSV-1. Treatment of mice with a topical application of AIU, 10% w/w, in a petrolatum-based ointment commencing 4 h post-infection did not significantly reduce the number of animals bearing distinct virus-induced lesions, nor the severity or the time for the lesions to heal when compared with infected control mice treated with ointment base. However, subsequent experiments with ^{125}I-labelled AIU in a petrolatum-based ointment applied to the shaved dorsal skin of rabbits showed that no significant absorption of the drug occurred (C.M. Walls, personal communication).

As an alternative vehicle for topical application of drug, a solution of AIU in DMSO was employed and its activity compared with IDU. AIU was not soluble directly in DMSO to any appreciable extent and was therefore prepared at its maximum solubility as a 10% w/v solution in 50% v/v DMSO in water. IDU, 40% w/v in DMSO, applied topically was effective in preventing infection in intradermally infected mice (Fig. 4a). Only five of 15

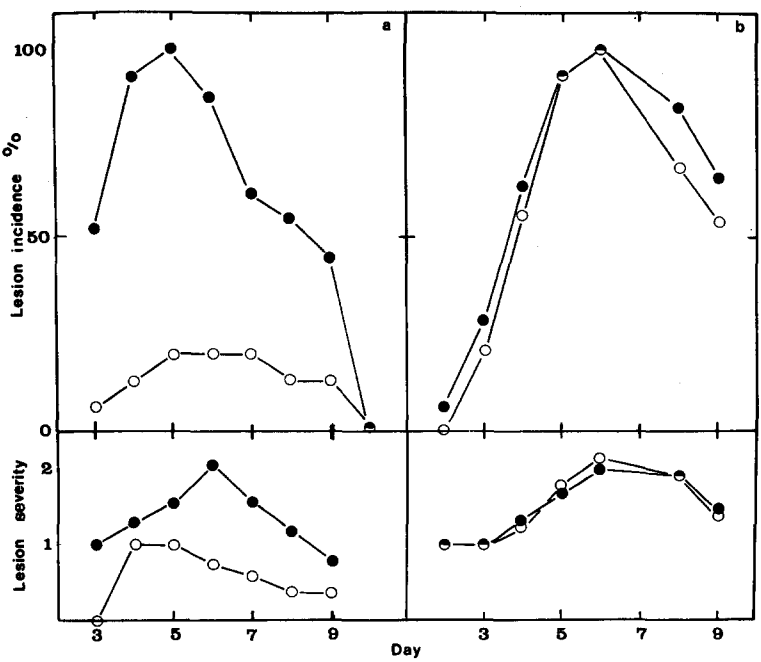

Fig. 4. Effect of IDU and AIU on HSV-1 skin infection in mice. Mice were infected intradermally and treated with drug (o), 40% IDU in DMSO (a) or 10% AIU in 50% DMSO in water (b) topically 4 h post-infection and twice daily for 4 days. •, Infected control. Animals were scored daily for the presence and severity of lesions.

IDU-treated mice showed signs of infection compared with 100% of DMSO-treated controls. The lesions of drug-treated mice were less severe than those of controls and were more transient, such that no more than three out of 15 showed evidence of infection on any one day. Using the same treatment regimen, AIU was ineffective by the criteria of incidence and severity of lesions (Fig. 4b). Moreover, the titres of HSV-1 recovered in homogenates of skin taken from the site of injection 5 days post-infection were considerably reduced by topical IDU treatment but not by AIU treatment (Table 2).

Since it was possible that DMSO was not a suitable delivery vehicle for AIU, two further experiments were performed in which mice were infected intradermally and treated with AIU, 500 mg/kg/dose, either as a solution administered subcutaneously or as a suspension in 0.5% carboxymethyl cellulose administered orally by gavage. Drug treatment had only a marginal effect on the number of mice which developed herpes skin lesions and the severity of those lesions (Fig. 5). As a consequence of drug treatment, 93% (subcutaneous AIU) and 87% (oral AIU) of mice developed lesions compared with 100% of controls ($P>0.05$). However, it was apparent that drug treatment delayed the development of lesions. Thus, the mean time from infection until the first appearance of lesions (d) was 4.2 days for mice treated with AIU by the subcutaneous route compared with 3.3 days for controls and the difference was significant ($P<0.05$). Treatment with AIU by the oral route also resulted in a significant increase in the mean time from infection until the first appearance of lesions (3.2 days in drug-treated mice compared with 2.4 days in controls ($P<0.05$)). Virus titres in the skin 5 days post-infection were also determined. In mice treated with AIU by the subcutaneous route the geometric mean titre ($10^{6.0}$ p.f.u./g tissue) was not significantly different from that of controls ($10^{5.9}$ p.f.u./g), nor did AIU treatment by the oral route significantly reduce virus in the skin ($10^{6.2}$ p.f.u./g) compared with controls ($10^{5.8}$ p.f.u./g, $P>0.05$).

TABLE 2

Effect of topical treatment with IDU or AIU on the titre of virus in mouse skin

Drug treatment	Virus yield (p.f.u./g tissue)		t test (P)
	Drug treated	Control	
40% IDU	$10^{5.5}$; $10^{4.1}$ $<10^3$ [a] ; $<10^3$ $<10^3$	$10^{7.5}$; $10^{6.9}$ $10^{6.9}$; $10^{6.9}$ $10^{6.6}$	<0.001
10% AIU	$10^{6.7}$; $10^{6.2}$ $10^{5.7}$; $10^{5.5}$ $10^{5.3}$	$10^{6.3}$; $10^{6.2}$ $10^{5.8}$; $10^{5.6}$ $10^{5.4}$	>0.05

[a] $<10^3$ = no virus detected in tissue homogenates at the lowest dilution tested.

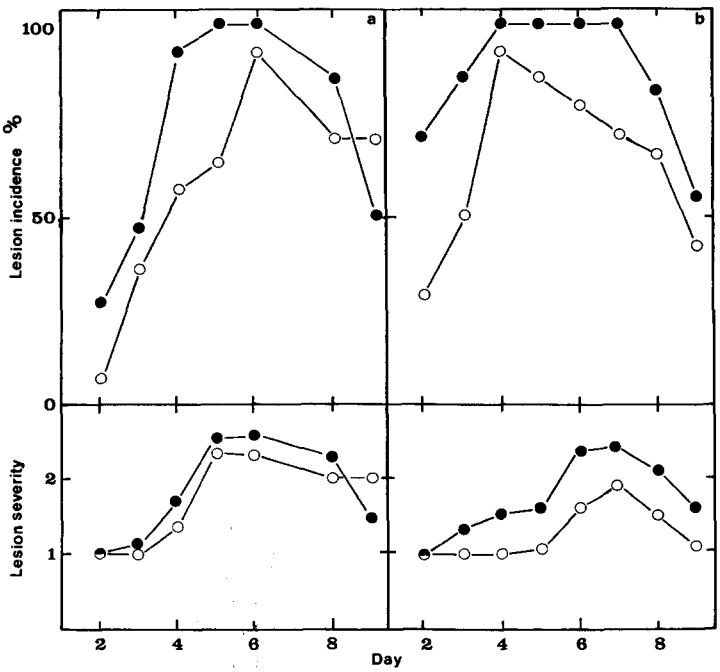

Fig. 5. Effect of AIU on HSV-1 skin lesions in mice. Mice were infected intradermally and treated with AIU (o) subcutaneously (a) or orally (b) 500 mg/kg/dose 4 h post-infection and twice daily for 4 days. •, Infected control. Animals were scored daily for the presence and severity of lesions.

DISCUSSION

The inhibition of growth of wild-type HSV-1 in cell cultures by AIU described here confirms the observations of Cheng et al. [5] and De Clercq et al. [10], who also observed that it was less potent than the parent compound IDU. Prusoff and coworkers have shown that AIU is specifically phosphorylated by the HSV-1-induced thymidine kinase [3] and as such is incorporated into newly synthesised virus DNA [4, 15]. They suggest that inhibition of virus replication is a direct consequence of the incorporation of the thymidine analogue into DNA [15]. The resistance of TK^- virus to AIU (Fig. 1) can thus be explained in terms of the absence in the infected cell cultures of the virus-induced thymidine kinase which is a key component in the intracellular processing of the drug. Co-cultivating TK^- virus with wild-type TK^+ virus in the presence of AIU resulted in a reduction in yield of both virus types (Table 1), demonstrating the inherent sensitivity of TK^- virus to AIU if the drug is first phosphorylated by the thymidine kinase of the co-infecting wild-type virus. However, the lack of antiviral activity of AIU in vivo is unlikely to be due to the virus immediately developing drug resistance by failing to express thymidine kinase. Our results (Fig. 3) show that wild-type virus is inherently susceptible to the antiviral effects of AIU in vivo, albeit in the localised environment of the peritoneal

cavity. Furthermore, it has been shown that, whilst TK^- mutants can be generated with relative ease in vitro, they cannot readily be obtained in vivo [13] and such mutants are less virulent in vivo than wild-type virus [14, 32].

The failure of AIU to exhibit a useful antiviral activity in vivo seems more likely to be related to its relative lack of potency as measured in cell culture systems (Fig. 1, and refs. 5, 10) and/or its bioavailability. Using topical administration of drug in experimental herpes keratitis of rabbits where problems of drug availability and metabolism are minimised, Albert and coworkers [1, 28] have reported AIU to be effective but less potent than the parent compound IDU. High concentrations of AIU in solution (8 mg/ml) or ointment (10%) were required to achieve a therapeutic effect equivalent to that obtained with IDU (1 mg/ml or 0.5% ointment). The relative insensitivity of HSV-1 to AIU may explain why others [19] have been unable to demonstrate antiviral efficacy in experimental keratitis. Indeed, using the model of Jones and Al-Hussaini [18] we also have been unable to show any significant therapeutic effect in herpes keratitis using a 10% AIU ointment (G.M. Scott and I.S. Sim, unpublished observations) with a wild-type strain of HSV-1 which exhibited a susceptibility to AIU in cell culture similar to that reported here.

Drug bioavailability following oral or parenteral administration may limit the efficacy of AIU but preliminary studies (T.J. Forest and C.M. Walls, personal communication) indicated that AIU is well absorbed from the gastrointestinal tract of rats and rhesus monkeys and studies with ^{125}I-AIU showed it to be widely distributed in tissues, except the brain and spinal cord, following oral or intravenous administration to rats. Failure to penetrate neural tissue may account for the lack of efficacy of AIU in systemically infected C3H mice which die of viral encephalitis. Our results show that IDU was effective in protecting mice from such a lethal infection (Fig. 2). HSV-1 infection of the mouse skin represents a useful alternative model to study drug efficacy, by-passing the requirement for drug to penetrate the central nervous system. When high doses of AIU were administered by the oral or subcutaneous routes, the peak titres of virus in the skin of WS mice were not reduced nor was the final outcome of the infection, lesion occurrence or severity, modified (Fig. 5). There was, however, a significant delay in the appearance of lesions following AIU treatment which may be explained in terms of an initial reduction in the rate of virus replication, thus delaying the establishment of the infection.

When applied topically to HSV-1 skin infection of mice, the efficacy of AIU might also be limited by its bioavailability. IDU, when applied at sufficiently high concentration in DMSO, penetrated the skin and reduced the titres of virus recovered from skin homogenates (Table 2), thus favourably affecting the course of virus infection restricting both the number of mice which developed lesions and the severity and duration of lesions (Fig. 4). Others [30] have found that IDU applied topically in DMSO was effective as a 40% solution but only marginally effective or ineffective as 10% and 5% solutions. The failure of AIU to dissolve in absolute DMSO and the need to use a solution of drug in 50% DMSO in water does not necessarily preclude its usefulness when applied topically. Penetration of the skin by a number of drugs is enhanced by the addition of DMSO to

aqueous solutions, but the effect is most marked when concentrations of DMSO are in excess of 70% (see refs. 20, 21 for review). Clearly, however, in the experiments reported here, insufficient AIU penetrated the skin when applied topically to affect virus replication or the development of herpetic lesions. The failure to demonstrate a significant antiviral effect for AIU applied topically either in ointment or DMSO is in marked contrast to the work of others [23] who reported significant reduction in virus titres at the site of infection and in lesion occurrence and mouse mortality. Park et al. [23] employed a high concentration of AIU in ointment (30%) and adopted a more intensive treatment regimen which together may have resulted in high concentrations of drug penetrating the tissues. In addition, the use of the lip as the site of infection may have permitted greater AIU absorption and also offered the possibility of the animals ingesting the drug during grooming. A surprising feature of the work of Park and coworkers is that they achieved a successful therapy when infecting with herpes simplex virus type 2 (HSV-2). It has been reported [27] that strains of HSV-2 are relatively more resistant to AIU than HSV-1 in vitro and specifically that the replication of nine strains of HSV-2 tested was only slightly or not at all inhibited by AIU at concentrations up to 400 μM. We have found (I.S. Sim, unpublished observations) that the inhibition of HSV-2 replication requires concentrations of AIU 10–20-fold greater than that required to achieve an equivalent reduction in the yield of HSV-1, results which are in general agreement with the observations of De Clercq et al. [10]. Clearly, the factors which influence the utility of the drug are complex. We have been unable to show any useful therapeutic effect for AIU in HSV-1 infections in vivo and attribute this lack of utility primarily to the relative lack of antiviral potency, as assessed in cell culture assay systems, compared with the parent compound IDU.

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