Successful therapy of herpes hominis keratitis in rabbits by 5-iodo-5'-amino-2',3'-dideoxyuridine (AIU): A novel analog of thymidine


The efficiency of 5-iodo-5'-amino-2',3'-dideoxyuridine (AIU) in the therapy of experimental herpes keratitis in rabbits has been examined. Virus infections were established bilaterally in 40 animals using herpes simplex, type 1 (NIH strain 11124). Twenty-four hours after infection the rabbits were divided into five matched groups of eight and each group was treated, double-blind, with topical drugs at four-hour intervals for a total of 72 hours. The solutions instilled were: (1) saline; (2) IdUrd, 1 mg. per milliliter; (3) AIU, 1 mg. per milliliter; (4) AIU, 4 mg. per milliliter; and (5) AIU, 8 mg. per milliliter. Each eye was examined daily for 12 days and graded independently by two ophthalmologists. Although IdUrd and AIU (8 mg. per milliliter) were effective therapeutically, IdUrd had a greater effect. The AIU at 1 and 4 mg. per milliliter were less active, but showed more rapid healing than the saline control. Viral recovery studies are consistent with the clinical observations. A second independent experiment, similar to that described above, gave essentially identical results. Although less potent than IdUrd, AIU does provide effective therapy for herpes keratitis.

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5-Iodo-5'-amino-2',3'-dideoxyuridine (AIU) (Fig. 1), a new thymidine analog, has been found to be a potent inhibitor of herpes simplex virus, type 1 (HSV-1) replication in cell culture.1-2 Herpes simplex virus, type II, is, however, resistant to the drug. In contrast to other antiviral nucleoside analogs, such as 5-iodo-2'-deoxyuridine (IdUrd), 5-trifluoromethyl-2'-deoxyuridine (FdThd), cytosine arabinoside (ara-C), and adenine arabinoside (ara-A), AIU exhibits little, if any, cellular toxicity.1-2 Preliminary studies suggest that AIU selectively inhibits viral specific DNA
The present report is a study of the effectiveness of AIU in the therapy of experimental herpes virus hominis keratitis in the rabbit.

Materials and methods

Experiment 1.

Drugs. IdUrd was obtained from Sigma Chemical Company, St. Louis, Mo. The synthesis and chemical properties of AIU will be described elsewhere (Lin, T.-S., Neenan, J. P., Cheng, Y. C., et al.: J. Med. Chem., in press). Drug solutions were prepared at the indicated concentrations in isotonic phosphate-buffered saline (PBS), pH 7.0, and were sterilized by filtration through 0.22 μ Millipore filters prior to use. Concentration was determined spectrophotometrically using a molar extinction coefficient of 7,500 at 287 nm. for both IdUrd and AIU. Since the hydrochloride salt of AIU had a significantly greater solubility than the free amine form (saturation solubility is 27 mg per milliliter at pH 6.5), all solutions contained AIU as its hydrochloride salt. Stock solutions were prepared as follows: AIU (free amine, 177 mg, 0.5 mmol.) was suspended in 10 ml. of distilled water and 0.55 ml. of 1 M HCl added slowly with stirring. When the AIU completely dissolved the solution was adjusted to 16 mg per milliliter. This stock solution was diluted to the indicated concentration using water and an appropriate volume of 2 x PBS. Although AIU is extremely soluble in aqueous solutions below pH 7.0 and above pH 9.3, between these pH values AIU has a saturation solubility of approximately 4.5 mg per milliliter.

Animals. A total of 40 New Zealand albino male rabbits weighing 1.5 to 2 kilograms each were used. The rabbits were maintained in special quarters with ventilation and isolation techniques designed for the maintenance of animals with infectious diseases.

Virus. The virus used was herpes simplex, type 1 (NIH strain No. 11124). Viral stocks were maintained through multiple passages in cultured Vero cells using an infection multiplicity of 10 plaque forming units per cell. Between passages of virus pool was stored at -60° C. in Hanks base minimal essential media supplemented with 50 per cent (V/V) fetal bovine serum. The frozen virus suspension was thawed to 21° C. and one-tenth milliliter of the virus solution containing 10° infectious units (TCID₅₀) was used for inoculation.

Inoculation. Both corneas of each animal were superficially abraded to a depth of about 0.2 mm. with a dulled 21-gauge needle. Two vertical and two horizontal scratches, each about 6 mm. in length, were made in the central corneal epithelium. Care was taken to avoid deep penetration of the stroma. One-tenth milliliter of virus-containing solution was dropped into the lower cul-de-sac of each eye. Following this the lids were manually closed and rubbed against the eye for 30 seconds.

Examination and grading. Each animal was graded daily by two ophthalmologists independently in the manner described below, and the average of the two grades was recorded. Grading was carried out for a period of nine days following cessation of treatment.

The corneas were examined daily using a loupe of 3 x magnification. The eyes were first observed using a focused hand held Tungsten examining light. Following this, a fluorescein solution (two drops of PBS added to a Fluor-i-Strip [Ayerst]) was instilled and the corneal changes evaluated under ultraviolet light. The severity of the keratitis was observed and graded on a scale of zero to three.

Grade 0: no detectable dendritic ulcers. Grade 0.5: one to four dendritic ulcers limited to epithelium along the lines of abrasion with no stromal involvement. Grade 1.0: five to nine dendritic ulcers limited to the epithelium along the lines of abrasion with no stromal involvement. Grade 1.5: five to nine dendritic ulcers which are not limited to the lines of abrasion with or without deep stromal edema beneath the involved epithelium. Grade 2.0: 10 to 20 dendritic ulcers, or a confluent ulcer not exceeding one-third of the surface area of the cornea, with or without deep stromal edema beneath the involved epithelium. Grade 2.5: > 20 dendritic ulcers with or without deep stromal edema beneath the involved epithelium. Grade 3:
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Fig. 2. Mean lesion score per eye as a function of time after infection. The 72-hour period during which drug therapy was applied is indicated by cross-hatching. The standard error of the mean is denoted by a vertical line (1).

a confluent corneal ulcer involving over one-third of the corneal surface with deep stromal edema beneath the involved epithelium.

The amount of discharge and conjunctival involvement was recorded daily.

**Treatment.** Twenty-four hours following viral infection the animals were divided into five groups which were matched with regard to severity of the lesions. Treatment was then initiated with the following five solutions:

1. Control—isotonic phosphate-buffered saline, pH 6.5;
2. IdUrd, 1 mg. per milliliter;
3. AIU, 8 mg. per milliliter;
4. AIU, 4 mg. per milliliter;
5. AIU, 1 mg. per milliliter.

The stock solutions were stored in opaque stock bottles identified by a letter (A through E). The opaque squeeze-type dropper bottles used in treating the rabbits were filled from these stock solutions. Neither the individuals treating the rabbits nor the ophthalmologists who graded the severity of the lesions knew the code until the experiment was terminated. Two drops of the respective solution were instilled into the lower cul-de-sac of each eye at four-hour intervals for a 72-hour period. Each animal received the same medication in both eyes.

**Viral studies.** Viral cultures were taken with a cotton-tipped applicator inserted into the lower fornix and rolled across the cornea into the upper fornix. The specimens were put in tissue culture fluid (minimum essential media plus 50 per cent fetal bovine serum) at 4° C. and then frozen at -60° C. Immediately before the assay, the vials were thawed and the cotton swabs were compressed three times in the media. Virus quantitation was done by inoculation of serial 10-fold dilution into four Vero cell cultures. These were observed for cytopathic effect on days 3, 5, 7, and 10 after inoculation. The endpoints were calculated by the method of Reed and Muench. While this procedure does not provide an absolute quantitation of the virus titer in the infected tissue, it does give a qualitative picture of the drugs' efficiency in reducing the general level of ocular virus.

**Experiment II.** In order to verify the findings of Experiment I, a second, totally independent, drug therapy experiment was undertaken. The experimental protocol and scoring system were as given above with the following modifications: (1) A total of 32 rabbits were divided into four groups of eight; (2) prior to HSV-1 infection, three overlapping circular abrasions were made on each cornea with a Treefine; (3) AIU was instilled at 7.7 mg. per milliliter in PBS solutions adjusted to (a) pH 6.5 and (b) pH 9.4. Since protonation of the 5'-amino group of AIU has a pK value of 7.8, drug instillation was done at two pH's to determine whether protonation would enhance or decrease AIU efficacy; (4) IdUrd (0.91 mg. per milliliter) was administered in PBS at pH 9.4.

**Results**

**Therapy of herpesvirus hominis keratitis (Experiment I).** By 24 hours following infection, all 40 rabbits had corneas which possessed between three and 15 small dendritic ulcers along the lines of the
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abrasion, with the average grade ranging from 0.9 to 1.1. Treatment was instituted at this time. The mean and cumulative corneal lesion grades (see Methods) observed during the 12-day observation period are shown in Figs. 2 and 3. Because the conjunctival changes and amount of discharge were extremely variable, the data were not considered sufficiently significant to report.

Saline treatment. These animals showed increasingly severe herpetic keratitis reaching an average severity of 2.3 by the fourth day after infection. The keratitis remained above grade 2.0 until the sixth day of infection. Then over a 24-hour period the degree of keratitis improved from 2.2 to 0.8. Residual keratitis persisted in some animals with the average being below grade 1 from days 7 to 12 after infection. Four animals in this group died with clinical findings consistent with encephalitis, the deaths occurring on days 2, 7, 9, and 11.

IdUrd-treated animals. The keratitis in these animals improved during the period of treatment going from an average grade 1 on the first day of treatment to 0.3 on the final day of treatment. One day following the cessation of treatment (i.e., day 4 after infection), 13 of the 16 eyes appeared entirely healed, with the average grade being 0.1. By 48 hours following the cessation of treatment (i.e., 6 days after infection) ulcers were present in 9 of the 16 eyes, with the average grade of keratitis having increased to 0.4. The animals in this group continued to manifest a low-grade keratitis ranging between 0.5 and 0.7 during the next five days of treatment (i.e., days 6 to 10 after infection). By day 12, the final day of grading, only two animals had dendritic lesions. Four animals in this group showed clinical signs suggestive of encephalitis; 1 animal died.

AIU-treated animals, 8 mg. per milliliter. At the start of treatment these animals had an average keratitis of grade 0.9. The keratitis showed little change during the 72-hour period of treatment with the average grade being 0.7 on the third day of treatment. At this time only three of the
16 eyes were entirely healed on clinical examination, the remainder ranging between grades of 0.5 and 2.0. The average grade of keratitis remained at approximately the same level at 24 and 48 hours following the cessation of treatment (i.e., grades of 1.0 and 0.7, respectively, on the fourth and fifth days after infection). Following this, healing ensued and by the seventh day of the experiment 11 of the 16 eyes were entirely healed. By the twelfth day of the experiment no residual lesions were seen. Five animals showed clinical signs of encephalitis during the course of the experiment; two of these animals died before the twelfth day.

**AIU-treated animals, 4 mg. per milliliter.** The keratitis in this group of animals worsened slightly during the 72-hour period of treatment, having advanced from a grade of 1.1 at time of treatment to 1.6 by the third day. Following cessation of treatment the severity of the corneal changes remained approximately the same until the seventh day after infection (four days following the cessation of treatment). At that time improvement paralleling that of the control group was seen with virtually complete healing occurring by the tenth day. Two animals in this group showed signs of encephalitis, but no deaths occurred during the twelve days of observation.

**AIU-treated animals, 1 mg. per milliliter.** These animals showed a similar response, during the period of treatment, to the group receiving 4 mg. AIU per milliliter. The average grade of keratitis on the date treatment was begun was 1.0. Following 72 hours of treatment the average of the gradation of the keratitis was 1.5. In the next 72 hours the keratitis worsened to a point where it approximated that seen in the control animals with the average grade on day 6 (three days after cessation of treatment) being 2.1. More rapid resolution followed than was seen in the controls and there was virtually total healing of the keratitis by day eleven. Six animals showed clinical symptoms of encephalitis in this group but no deaths occurred during the 12 days of observation.
Viral recovery studies. The relative concentrations of virus present in the cornea and fornices of the five groups of infected rabbits at the third day after infection (i.e., after 72 hours of treatment) followed in a general way the pattern of the clinical observations. The amount of virus recovered from the AIU (8 mg. per milliliter) and the IdUrd-treated eyes, although significantly lower than that from the control, were not significantly different from each other (Fig. 4). The amount of virus found after three days of therapy among the three groups treated with AIU was directly related to the concentration of AIU instilled into the eye. There was no significant difference between the amount of virus recovered in the eyes of the control animals relative to that found in the eyes of the rabbits receiving the lowest level of AIU (1 mg. per milliliter). The virus titers found in the other two groups of rabbits receiving the two higher levels of AIU (4 and 8 mg. per milliliter) were significantly different from the control. The statistical analysis was performed by an analysis of variance. No specimens were virus negative during the therapy period.

Experiment II. A second experiment was performed, with the modification described in Methods above, in order to provide a totally independent approach to the evaluation of the efficacy of this agent in the therapy of experimental herpesvirus hominis keratitis. The results of this experiment, expressed in Fig. 5 as cumulative lesion grades, were essentially identical to those previously obtained. While AIU, at both pH 6.5 and 9.4, exhibited significant improvement over the untreated control groups, IdUrd was more effective than AIU. The pH at which AIU was administered did not appear, however, to markedly influence its biological activity.

The data up to day 6 were analyzed nonparametrically with the Mann-Whitney test statistics for two independent samples. The lesion scores for both eyes of any one rabbit were summed. The following relationships were found, based on eight rabbits in each group, with the exception of the IdUrd-treated group which had
seven rabbits, because of death of one rabbit in this group on day 2. Each of the three drug-treated groups receiving either IdUrd or AIU was significantly different from that of the buffer-treated control group.

Discussion

The generally accepted therapy for acute herpes simplex keratitis at present includes the use of IdUrd (idoxuridine). This compound was originally synthesized by Prusoff, and the first successful treatment of human dendritic keratitis was reported by Kaufman, Martola, and Dohlman. Although the clinical value of IdUrd has been well established, there is a need for alternative antiviral therapy for ocular herpetic infections. IdUrd-resistant strains of HSV type 1 have been found. In addition, IdUrd exhibits significant cellular toxicity. This is manifested in undesirable side effects such as the development of follicular and papillary conjunctivitis, epithelial punctate keratopathy, and even occlusion of the punctum. Moderate to severe toxicity of the regenerating epithelium has been observed during corneal wound healing. IdUrd also inhibits stromal repair and decreased the strength of healing wounds, apparently as the result of diminished collagen content. How IdUrd affects the collagen content is not clear.

McGill and co-workers, during an assessment of the toxicity of IdUrd in the therapy of herpetic keratitis in man, report that the earliest signs of toxicity in the use of F,dThd are punctate epithelial erosions and epithelial microcysts which on continued therapy produce frank epithelial edema.

An additional concern is the teratogenicity of IdUrd. This has been demonstrated in newborn rats following systemic administration, and in pregnant rabbits receiving the drug topically to the eye in doses similar to those used clinically in humans. The toxicity and teratogenicity of IdUrd appear related to its incorporation into host DNA. A desirable characteristic for an alternative antitherpeptic drug would be a mode of action not involving incorporation into host DNA.

In the search for additional nucleosides effective against HSV, three of the most widely studied candidates have been ara-C, ara-A, and F,dThd. It is apparent, however, that these compounds share at least some of the undesirable characteristics of IdUrd.

AIU is a new thymidine analogue differing from IdUrd by the substitution of an amino group for the 5' hydroxyl. AIU, like IdUrd, inhibits the replication of herpes simplex virus, type 1 in vitro, however, AIU does not inhibit herpes simplex virus, type 2, in vitro, nor does it affect the replication of a variety of other DNA and RNA viruses, including vaccinia, adenovirus, measles, and Rous sarcoma virus (Prusoff and Ward, unpublished results). While HSV-2 ocular infections are clinically rare, the specificity of AIU for HSV-1 may be considered by some to be a practical limitation. However, at molar concentrations of AIU, F,dThd, ara-C, ara-A, and IdUrd which produced a comparable degree of inhibition of the replication of HSV in cell culture, only AIU produced no cytotoxicity in the uninfected host cell. This lack of cytotoxicity has been demonstrated in a variety of mammalian cells in culture including those derived from human, monkey, mouse, hamster, and the chicken. AIU, unlike IdUrd, produced no detrimental effect to growth when administered to suckling mice intraperitoneally in comparable amounts. Preliminary teratological studies show no ocular or systemic abnormalities in newborn mice treated with high doses of AIU; whereas, cataract, retinal dysplasia, and retardation of retinal development were observed when comparable doses of IdUrd were given (Albert, et al., unpublished results).
The mechanism of action of AIU is under investigation. Preincubation of HSV with AIU prior to infection in vitro did not decrease virion infectivity. When AIU is present in the media only during the absorption process, no inhibition of virus production occurs. Addition of AIU (200 μM) four to six hours after infection, markedly reduces the yield of progeny virus. Thus it appears that the critical time of action of AIU is at the time of HSV replication within the host cell. This has been confirmed by addition or removal of drug at various times after infection.

Significantly, AIU does not inhibit the rate of RNA or protein synthesis in either uninfected or HSV-infected Vero cells. AIU markedly inhibits the uptake of [14C] thymidine into the DNA of HSV-infected Vero cells; however, no inhibition of DNA synthesis is observed in uninfected Vero cells. In vitro experiments indicate that AIU inhibits cellular thymidine kinase but not the comparable enzyme induced by HSV-1. Metabolic studies have shown that AIU labeled with [35S] is transported into Vero cells only when infected with HSV-1, and is phosphorylated by a cell-free extract only from HSV-infected cells. The selective phosphorylation of AIU and its subsequent incorporation into infected cell DNA could account for its specific antiviral activity in the absence of host toxicity.

The observed biological properties of AIU do not result from deiodination or deamination of the analog. Deiodination would result in the formation of 5'-amino-2',5'-dideoxy-5-iodouridine which is a biologically inert compound. Hydrolytic deamination, if it were to occur, would result in the formation of IdUrd, however, the failure to observe typical IdUrd cellular toxicity does not support this hypothesis.

The present experiments show that AIU not only prevents the replication of HSV in vitro, but also is effective in the therapy of HSV keratitis in rabbits. The apparent lack of toxicity and teratogenicity of AIU, its greater solubility relative to IdUrd, and its specificity for HSV type 1 suggest that its continued investigation as a potential drug in the treatment of HSV keratitis in man is justified.

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