Effective Treatment of Experimental Herpes Simplex Keratitis with New Derivative, 5-Methylamino-2′-Deoxyuridine (MADU). (30224)

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Various analogues of the base groups of nucleic acid prepared for studies of chemotherapy of cancer have been found to exhibit activity against deoxyribonucleic acid (DNA)-containing viruses such as herpes simplex, vaccinia, and adenovirus(1-6). One such compound, 5-iodo-2′-deoxyuridine or IDU has been studied extensively in relation to suppression of herpes simplex virus(1,7-9). The compound functions as an analogue of thymidine by virtue of addition of iodine in the 5 position to the uridine. Being an analogue, IDU interferes with the utilization of thymidine by host cells as well as by virus and, in addition, it may incorporate(3,10,11) into their nucleic acids giving a fraudulent and likely nonfunctional DNA.

IDU attracted considerable attention when Kaufman(1,7,9) reported it to be highly effective for treating superficial corneal lesions caused by herpes simplex virus. Most, but not all, investigators(12) have reported success in use of the compound for treating herpetic keratitis, a disease which exhibits a highly variable course. The drug apparently stops proliferation of cells as well as virus. Even if nonlethal for cells, IDU carries some hazard through incorporation into cellular DNA resulting in sensitization to ultraviolet light and to X-ray(10,11). Genetic mutations might also result from such incorporation and this might conceivably bring about neoplastic transformation of cells.

Studies in our laboratories have been oriented toward finding a compound which would be therapeutically active against herpes simplex virus but which would neither incorporate into cellular DNA nor contain a halogen group. In the studies, 5-methylamino-2′-deoxyuridine (MADU), was found active *in vitro* in suppressing herpes simplex infections and to exert a highly beneficial effect for prophylactic and therapeutic treatment of herpes simplex virus infection in the rabbit eye. MADU was first synthesized by Visser *et al*(13).

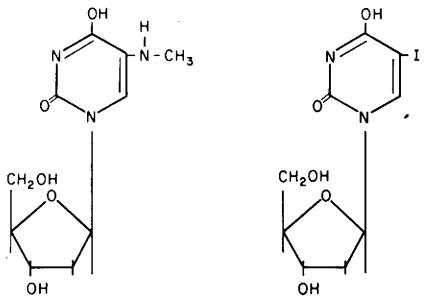


FIG. 1. Chemical structures of MADU and IDU.

The present report presents findings in comparative studies of MADU and IDU *in vitro* and for treating experimental herpetic keratitis. The chemical structures of the two compounds are shown in Fig. 1.

Materials and methods. Virus. Herpes simplex strain McIntyre was used in the *in vitro* tests. The virus was propagated and titrated for infectivity in grivet monkey kidney cell cultures (GMK). Herpes simplex strain HF 378 was employed in the *in vivo* tests in rabbit cornea. This strain was chosen for its apparent lack of neurotropism for rabbits and for its failure to cause symptoms of central nervous system infection or death following inoculation onto the scarified cornea. The virus was propagated in 7-day-old embryonated hens' eggs which were inoculated *via* the yolk sac and from which the infected allantoic fluids were collected as source of virus for rabbit inoculation purpose. The lethal infectivity titer of such seed stock was $10^{-5.3}$ per 0.2 ml when tested in 7-day-old embryonated hens' eggs.

Compounds. The 5-methylamino-2′-deoxyuridine (MADU) was synthesized in the Merck Sharp & Dohme Research Laboratories by Drs. T. Y. Shen and B. O. Linn as part

of a cooperative program designed to search for useful antiviral substances, to be recorded elsewhere. The method of Ueda(14) was employed for synthesis. Crystalline 5-iodo-2'-deoxyuridine (IDU) was purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. Solutions of the compounds were prepared for ophthalmic use in physiological saline solution by Drs. T. Macek and A. Marcus of these laboratories. All solutions were sterilized by filtration.

In vitro *tests*. Initial solution and serial 2-fold dilutions of the compounds were prepared in medium 199 containing 2% agamma calf serum. An amount of herpes simplex virus (McIntyre) was added to each dilution of drug so that 10 $TCID_{50}$ (50% tissue culture infectivity doses) were present in 1 ml. The virus-drug mixtures were inoculated in quadruplicate in 1 ml amount into 6- to 7-day-old roller tube cultures of GMK. A similar series of preparations of drug without added virus was prepared for simultaneous assay of toxicity. Simultaneous titration of viral infectivity was also made to establish virus dose which was actually employed in the tests. The cultures were incubated stationary at 37°C and observed for specific cytopathic effect caused by herpes virus or for toxic destruction of cells by drug. All experiments were terminated about the tenth day following inoculation when the virus cytopathic action was complete. The toxicity titer is the least amount of compound which caused toxic deterioration of the cells. The antiviral titer is the least amount of drug which caused total suppression of viral destruction of the cells. The antiviral index is the ratio of the toxicity titer divided by the antiviral activity titer of the drug. *In vivo*. The right cornea of healthy New Zealand rabbits weighing 4 to 5 lb was anesthetized with 0.5% solution of proparacaine HCl, abraded lightly with the point of a sterile 25 gauge hypodermic needle, and inoculated with 0.2 ml of HF 378 strain herpes simplex virus containing about 5000 egg lethal doses of virus. This virus dose caused moderate to severe dendritic lesions on the cornea by the third day following inoculation. Drug was administered to the infected eyes in 2-drop amounts given with an eye dropper according to the regimens and in the numbers of animals described in the text. Infected but untreated animals were given physiological saline solution by the same regimen and observed for control purpose. Selection of animals to receive drug or to serve as control was at random and each drug was identified by code so that the investigator was unaware of what compound was being given. Observations were also made without prior knowledge of the kind of treatment. The eyes were observed daily for development of lesions following instillation of a 0.2% sterile fluorescein solution. The excess fluorescein was removed by irrigation with sterile Dacriose solution and the extent of pathology was scored according to a numerical system reflecting the percentage of the cornea involved, *viz.*, 4+ grading down to ± and zero. The mean score for all the animals in each group was calculated for each observation period. This provided a numerical expression by which the extent of pathology caused by herpes could be compared among the groups. All drugs were checked for irritancy in rabbit eye in the concentrations and under the regimens used. In no instance was there evidence of any significant irritation to the bulbar or palpebral conjunctivae or to the cornea by either of the compounds.

Results. In vitro *tests*. Table I presents the findings in the *in vitro* tests to compare the effectiveness of MADU and IDU. Both drugs were of relatively low apparent toxicity

TABLE I. Comparison, *in vitro* Activity, of MADU with IDU Against Herpes Simplex Virus in Grivet Monkey Kidney Cell Cultures (GMK).

Exp No.	Compound	Titer in γ/ml* Toxicity for GMK	Anti-herpes simplex virus	Antiviral index, toxicity/antiviral
1	MADU	>5000	2.5	>2000
	IDU	2500	20	125
2	MADU	> 450	<7.3	> 62
3	IDU	2500	20	125
4	IDU	1380	10.6	130

* Least concentration of drug causing toxic destruction of the cells and least concentration of drug affording protection against herpes simplex virus infection.

for the resting GMK cells. The antiviral activity of MADU was at least as great as that of IDU under the conditions of the test. In vivo *tests*. Studies were carried out in rabbits to compare the therapeutic and prophylactic-therapeutic effectiveness of MADU and IDU in treating herpes keratitis in rabbits. Fig. 2 presents the findings in a trial to evaluate therapeutic efficacy when drug was started 3 days after virus was given and at a time when there was extensive gross corneal pathology due to herpes virus. Both drugs were given in 0.1% concentration. By one regimen, drug was given at hourly intervals for 8 hours per day and by another, the drug was given for 12 hours per day. There was spontaneous regression in eye pathology in the control group. However, the rate for such regression was greatly enhanced by administration of either drug. IDU given on a 12-hour regimen appeared to be most effective while MADU given on an 8-hour regimen was least effective. IDU given for 8 hours compared roughly in effect with MADU given for 12 hours. The rate for total cure, *i.e.*, freedom from detectable lesions on the cornea, as noted on the eighth day following virus, provided additional information for comparison. None of six animals which received saline placebo was cured and only 1 of 6 animals given MADU on the 8-hour regimen was free of gross lesions. By contrast, nearly all animals given IDU and those given MADU at 12 hourly intervals were cured.

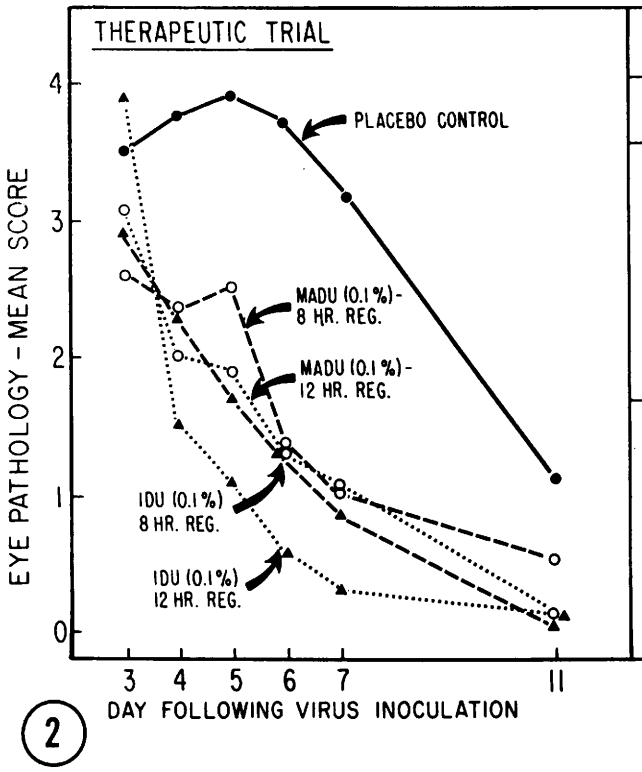
In a similar experiment, with findings shown in Fig. 3, the effectiveness of greater concentrations of MADU, *i.e.*, 0.25% and 0.5%, were compared with that of 0.1% IDU. The drugs were started on the third day after virus and were given hourly for 8 hours per day. The rate of regression of eye lesions in the rabbits given MADU in the higher concentrations was roughly equal to that of IDU given in lesser amount. None of the controls was cured by day 7. The cure rates for the drug treated groups were roughly the same within the range of variation shown.

In a third experiment (Fig. 4) the drugs were started before virus was given in order to measure the effect of the drugs when treatment was started before virus (prophylactic) and continued after virus inoculation (therapeutic). It is seen that the virus was almost totally suppressed. The lesions on the cornea were minimal and did not progress further although a majority of animals still exhibited some degree of eye pathology on the seventh day following virus. The drugs were roughly equal in efficacy, considering the variations in the test method.

Discussion. Synthesis of 5-methylamino-2′-deoxyuridine (MADU) was first reported by Visser *et al*(13) but no tests for antiviral activity have been recorded. The present findings indicate a remarkable degree of activity of MADU against herpes simplex virus infection when tested *in vitro* in resting grivet monkey kidney (GMK) cells and when applied topically *in vivo* in experimental herpetic keratitis in the rabbit. Such beneficial effect was obtained in herpetic keratitis even though the drug was given only during an 8- or 12-hour period each day for 4 or 5 days. The antiviral activity *in vitro* and the antiviral index of MADU roughly approximated that for 5-iodo-2′-deoxyuridine (IDU) which was included for comparative purpose. In the simulated clinical tests carried out in rabbits, MADU given in 0.5% or 0.25% concentration was only slightly less active in supressing ocular herpetic infection than was IDU in 0.1% concentration, the near limit of solubility of this compound. The MADU revealed no apparent gross conjunctival or corneal toxicity under the conditions of administration.

Tests of combined prophylactic-therapeutic efficacy were performed to develop data on which a judgment could be rendered as to possible benefit for initiating drug therapy in man immediately prior to or in the very early period after herpes virus infection. In spite of the extreme severity of the experimental herpes simplex virus challenge, IDU and MADU performed extremely well in suppressing development of herpetic keratitis. MADU in 0.25% concentration appeared to be slightly more effective than IDU in 0.1% solution.

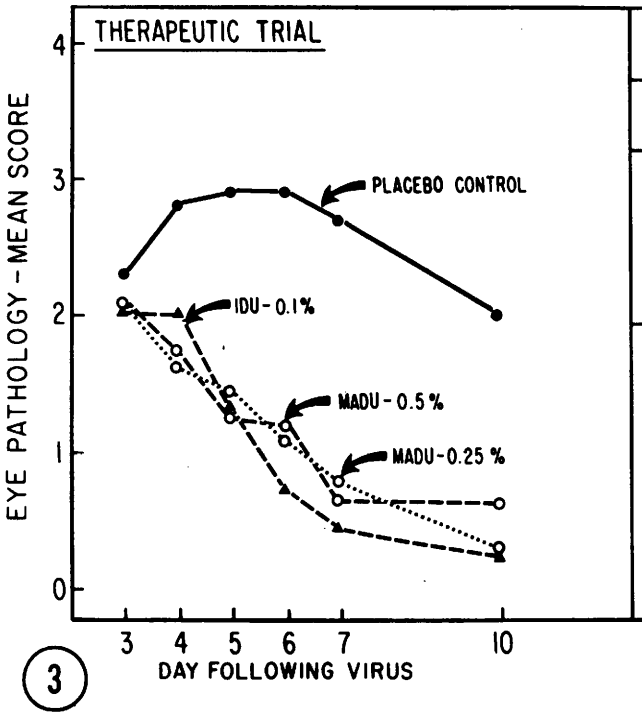
Chemotherapeutic treatment of herpetic



CURE RATE: DAY 8 FOLLOWING VIRUS	
DRUG	NO. CURED / TOTAL
PLACEBO	0/6
MADU 0.1 % 8 HR. REGIMEN	1/6
MADU 0.1 % 12 HR. REGIMEN	5/6
IDU 0.1 % 8 HR. REGIMEN	6/6
IDU 0.1 % 12 HR. REGIMEN	5/6

TREATMENT:
DRUG STARTED - DAY 3 POST VIRUS
DRUG CONTINUED - 4 CONSECUTIVE DAYS.
DRUG GIVEN HOURLY:
8 HR. REGIMEN - 8 HOURS PER DAY
12 HR. REGIMEN - 12 HOURS PER DAY

2



CURE RATE: DAY 7 FOLLOWING VIRUS	
DRUG	NO. CURED / TOTAL
PLACEBO	0/8
MADU 0.5 %	2/8
MADU 0.25 %	3/6
IDU 0.1 %	6/9

TREATMENT:
DRUG STARTED - DAY 3 POST VIRUS.
DRUG CONTINUED - 4 CONSECUTIVE DAYS.
DRUG GIVEN HOURLY - 8 HOURS PER DAY.

3

FIG. 2. Evaluation of MADU and IDU for therapeutic treatment of experimental herpes simplex infection in rabbit eye using different regimens.
FIG. 3. Evaluation of MADU and IDU for therapeutic treatment of experimental herpes simplex infection in rabbit eye using different drug concentrations.

keratitis in man reasonably justifies a risk since the natural disease may be extremely progressive and blindness may result. IDU has generally proved beneficial for treating superficial herpetic keratitis but of considerably less or even adverse effect in cases with stromal involvement(15). The drug apparently stops proliferation of cells as well as virus and such apparent corneal healing as occurs during treatment might result from sliding and cell migration from adjoining normal epithelium(15,16). Healing might be more significantly impaired by IDU in the deep stroma where cell migration is much slower.

Aside from suppression of stromal healing (15,16) application of IDU carries certain additional risks which presently exist only in theory. The compound has been demonstrated to incorporate(1,10,11) into host cell DNA, replacing thymidine, and this conceivably might lead to mutations of a possible oncogenic nature. Treatment of human cells with the bromo derivative has been demonstrated to cause detectable chromosomal changes(17). Additionally, incorporation of the halogen-containing compound may cause sensitization to ultraviolet light and X-ray(10,11).

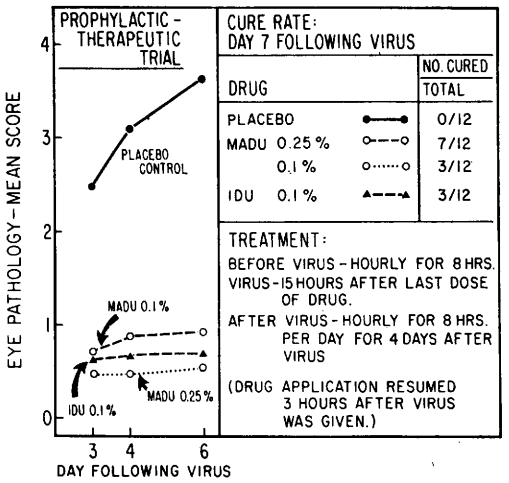


FIG. 4. Evaluation of MADU and IDU for prophylactic-therapeutic treatment of experimental herpes simplex infection in rabbit eye.

MADU contains no halogen and would be free of any deleterious effect from such molecule. Visser *et al*(13) suggested in their original report that MADU might incorporate into the DNA of host cells. Such possibility has recently been investigated in these laboratories by Dr. R. W. Burg (unpublished). The preliminary findings have shown that if incorporation of MADU does occur, the extent is less than 10% of that of either IDU or thymidine.

The freedom of toxicity of MADU for rabbit eye at the concentrations used, the absence of halogen, the apparent lesser incorporation than IDU into host cell DNA, and the high level of activity justify clinical evaluation of the compound in those situations in which IDU would normally be indicated. The various properties of MADU, as stated above, suggest a possible margin of safety and consequently greater ability than IDU if the efficacy of MADU in clinical suppression of herpetic lesion in man is comparable with that of IDU.

Summary. A recently synthesized compound, 5-methylamino-2′-deoxyuridine (MADU) was shown to be highly active in suppressing infection with herpes simplex virus *in vitro* and to exert beneficial effect in therapeutic and prophylactic-therapeutic treatment of experimental keratitis in the rabbit eye caused by herpes simplex virus. The beneficial effect was equal to or only slightly less than that observed in comparative tests with 5-iodo-2′-deoxyuridine (IDU). The low toxicity of MADU for the rabbit eye, the high level of activity, the absence of halogen, and the apparent low level incorporation into the DNA of host cells justify experimental investigations in man in infections in which IDU would normally be employed. The practical and theoretical implications of use of MADU and IDU in the human being are discussed.

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A New Method of Bandaging Orthotopic Murine Skin Grafts.* (30225)

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The technique of Billingham and Medawar(1) is the most widely employed method of orthotopic murine skin transplantation. It is simple to perform and consistently yields rapid and firm healing of the graft to its bed. However, because of the occlusive Plaster of Paris dressing, the graft is inaccessible to continuous or even repeated observation during the first 6 postoperative days.

Algire and Legallais(2), Edgerton and Edgerton(3), and Sabet *et al*(4) each designed a transparent chamber which permitted continuous observation of orthotopic murine skin grafts. Although these techniques yielded excellent results, the chambers were relatively difficult to apply (Sabet's technique requires 30 minutes per animal), and were therefore not suitable for routine use in skin transplantation.

The technique of bandaging presented herein affords continuous observation and/or manipulation of experimental skin grafts. It differs from the technique of Billingham and Medawar(1) in two respects: a) the vaselinized tulle is discarded and in its place a glass chamber overlies the graft bed, and b) the Plaster of Paris dressing is replaced with a self-adherent bandage.

Method. Six-week-old CFW male mice (Carworth Farms, New City, N. Y.) were grafted with 6-week-old DBA-1/J male mouse skin (Jackson Memorial Laboratory, Bar Harbor, Me.) according to the method of Billingham and Medawar(1): The right lateral thoracic wall skin was clipped and shaved. Suprapannicular square graft beds were prepared by dissection with curved scissors, and pinch grafts were applied onto them.

The bed was covered by a glass tube which was three-eighths inch tall and one-half inch in diameter (Fig. 1). The tube's base was flanged, describing an arc of curvature of one-half inch. Its upper opening was covered with a circular siliconized coverslip, which was sealed onto the chamber with Permount (Fischer Scientific Co., Fair Lawn, N. J.); and its flanged rim was covered with Dermicel Surgical Tape (Johnson and Johnson, New Brunswick, N. J.). At the time of operation Mastisol (400 g mastic gum, 12.5

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