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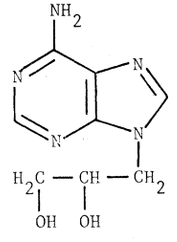
(*S*)-9-(2,3-Dihydroxypropyl)adenine: An Aliphatic Nucleoside Analog with Broad-Spectrum Antiviral Activity

Abstract. *(S)-9-(2,3-Dihydroxypropyl)adenine, a novel nucleoside analog, the sugar moiety of which is replaced by an aliphatic chain, inhibits the replication in vitro of several DNA and RNA viruses, including vaccinia, herpes simplex (types 1 and 2), measles, and vesicular stomatitis. It is also effective in vivo in reducing the mortality rate of mice inoculated intranasally with vesicular stomatitis virus.*

Besides interferon and its inducers, very few chemicals have been found to exhibit broad-spectrum antiviral activity. An example is ribavirin (1-β-D-ribofuranosyl - 1,2,4 - triazole - 3 - carboxamide), which was shown to inhibit the multiplication of a large variety of RNA and DNA viruses in both cell cultures and animals (*1*). Yet the antiviral action of ribavirin cannot be considered very specific, since it was found to inhibit DNA and RNA synthesis of the host cell at concentrations that coincided quite well with those at which it inhibited virus multiplication (*2*). Furthermore, ribavirin has been shown to exert both immunosuppressive and antitumor effects (*3*), to inhibit immune-complex glomerulonephritis in NZB/W mice (*4*), and to induce congenital anomalies when administered to pregnant hamsters (*5*).

We describe here the broad-spectrum antiviral activity of (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], an aliphatic nucleoside analog, which inhibited the replication of a number of DNA and RNA viruses (vaccinia, herpes simplex, vesicular stomatitis, and measles) at concentrations at which cellular DNA and RNA synthesis were not affected.



(S)-DHPA

(*S*)-DHPA belongs to a class of novel nucleoside analogs, the sugar moieties of which are replaced by aliphatic chains. Although some aliphatic nucleoside analogs (for example, aristeromycin, eritadenine, and willardiin) occur in nature (*6*), such substances are not normally involved in either nucleic acid biosynthesis or catabolism. In their "D-glycero" [or (*S*)-enantiomeric] form, the 2,3-dihydroxypropyl derivatives of uracil and adenine imitate the conformation of the β-D-ribonucleosides (*6*): their circular dichroism spectra are similar to those of the natural ribonucleosides; their 2′,3′-cyclic phosphates are split by (some) ribonucleases, and their aminoacyl esters effectively inhibit the peptidyltransferase reaction (*7*).

Table 1. Antiviral activity of (*S*)-DHPA in cell culture. The (*S*)-DHPA was added 1 hour after the cells had been inoculated with ~ 100 times the virus dose needed to infect 50 percent of the cells (~ 100 $CCID_{50}$). The antiviral activity of (*S*)-DHPA is given as ID_{50}; that is, the dose inhibiting the cytopathogenic effect (CPE) of the virus by 50 percent. The CPE was recorded as soon as it reached completion in the untreated virus-infected cell cultures; in this way it was also possible to detect a delay in the CPE. Abbreviations: PRK, primary rabbit kidney; HSF, human skin fibroblast; GMK, green monkey kidney; Vero, a continuous line of green monkey kidney cells; and BHK, a continuous line of baby hamster kidney cells.

Virus	Cell culture	ID_{50} (μg/ml)
DNA viruses		
Vaccinia	PRK	10–20
Vaccinia	HSF	10–20
Herpes simplex 1 (strain KOS)	PRK	10
Herpes simplex 1 (strain KOS)	HSF	20
Herpes simplex 2 (strain 333)	PRK	4–10
Herpes simplex 2 (strain 333)	HSF	7–20
RNA viruses		
Vesicular stomatitis	PRK	7–10
Vesicular stomatitis	HSF	2– 7
Vesicular stomatitis	HeLa	>200
Poliovirus 1	HSF	>200
Poliovirus 1	HeLa	>200
Coxsackievirus B4	HeLa	>200
Coxsackievirus B4	Vero	>200
Measles	Vero	4–40
Newcastle disease	HSF	>200
Newcastle disease	GMK	>200
Sindbis	BHK	>200

The antiviral activity of (*S*)-DHPA was explored in several cell cultures with several viruses (see Table 1). After the cells were inoculated with one of the viruses, they were exposed to various concentrations of (*S*)-DHPA. For each virus-cell system, we determined the dose of (*S*)-DHPA required to suppress viral cytopathogenicity by 50 percent. As shown in Table 1, several viruses, including vaccinia, herpes simplex (types 1 and 2), measles, and vesicular stomatitis, were inhibited by (*S*)-DHPA. Others such as poliovirus, Coxsackievirus, and Sindbis were not.

That the inhibitory effects of (*S*)-DHPA on virus-induced cytopathogenicity actually reflected inhibition of virus multiplication was determined by measuring virus growth in human skin fibroblast cultures that had been inoculated with vesicular stomatitis virus (VSV) and subsequently exposed to (*S*)-DHPA (Fig. 1A). The (*S*)-DHPA (100 μg/ml) caused a dramatic decrease of virus titer. This reduction amounted to approximately 4 $\log_{10}$ for the virus yields measured at 24 and 48 hours after infection (Fig. 1A).

The potential activity of (*S*)-DHPA in vivo was assessed in mice inoculated intranasally with VSV. This experimental infection resembles certain natural infections in humans (such as poliomyelitis, rabies, and herpetic encephalitis) in that the virus spreads from a respiratory tract site (olfactory mucosa) through the (olfactory) nerves to the brain. The intranasal VSV model was employed previously to evaluate the prophylactic and therapeutic efficacy of interferon and its inducers (*8*). Repeated doses of (*S*)-DHPA (2 mg per mouse or ~ 135 mg/kg) injected intraperitoneally 1 hour and 1, 2, 3, and 4 days after VSV challenge brought about a significant increase in the final number of surviving mice (Fig. 1B): 67 percent for the (*S*)-DHPA–treated mice compared to 37.5 percent for the control group ($P < .05$, chi-square test with Yates' correction). When the numbers of survivors were compared 9 days after infection, the difference between the (*S*)-DHPA group and the control group was significant at $P < .005$. Repeated doses of (*S*)-DHPA at 0.08 mg per mouse (~ 5.4 mg/kg) did not confer protection, whereas repeated doses at 0.4 mg per mouse (~ 27 mg/kg) gave slight protection (the final numbers of surviving mice were 55 percent for the treated group and 37.5 percent for the

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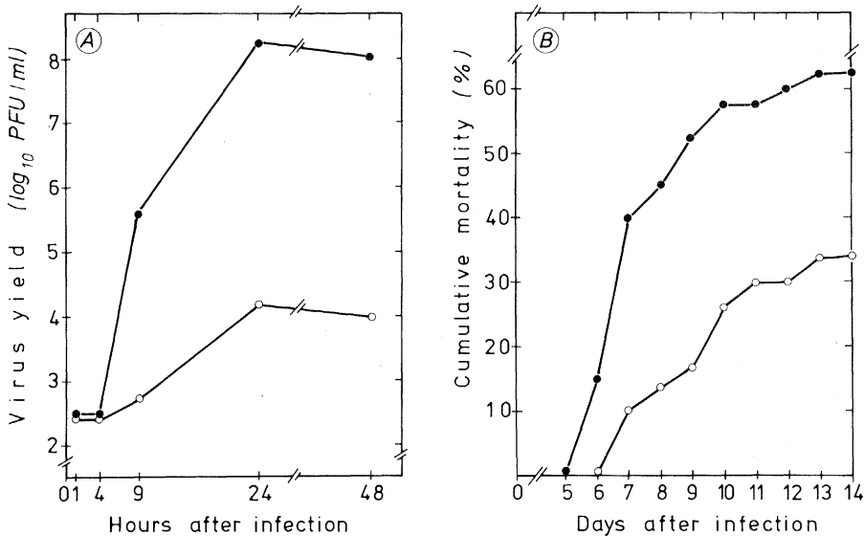

Fig. 1. Effect of (*S*)-DHPA on vesicular stomatitis virus multiplication in human skin fibroblast (HSF) cultures (A) and mice (B). (A) Confluent monolayers of HSF cells in plastic petri dishes were inoculated with vesicular stomatitis virus (4.5 $\log_{10}$ $CCID_{50}$ in 0.5 ml per petri dish) for 1 hour at 37°C and were then immediately exposed to (*S*)-DHPA (100 μg/ml). The cell cultures were frozen at −70°C at the times indicated on the abscissa and the cell homogenates were assayed for virus content by plaque formation (*PFU*, plaque-forming units) in mouse L-929 fibroblast cultures. (●) Control, (○) (*S*)-DHPA. (B) Twenty-day-old female NMRI mice (weighing ~ 15 g) were inoculated intranasally with vesicular stomatitis virus (2.5 $\log_{10}$ $CCID_{50}$ in 0.01 ml per mouse). (*S*)-DHPA was injected intraperitoneally at 2 mg per mouse (~ 133 mg/kg) 1 hour and 1, 2, 3, and 4 days after virus infection. Deaths were recorded daily. No deaths were noted beyond day 14 after infection. (●) Control (N = 40), (○) (*S*)-DHPA (N = 30).

control group; data not shown). No signs of toxicity were noted in mice that were injected with (*S*)-DHPA (for three consecutive days) at 1 g/kg either intraperitoneally or intravenously.

Various other aliphatic nucleoside analogs were examined in virus-cell systems in which (*S*)-DHPA exhibited marked antiviral activity: primary rabbit kidney cells with VSV, vaccinia, and herpes simplex 1 (strain KOS) (see Table 1). Only the (*S*)-enantiomer of DHPA proved active. The (*R*)-enantiomer, (*R*)-DHPA, was not. The racemic mixture, (*R,S*)-DHPA, was almost as effective as the (*S*)-enantiomer. Whereas (*S*)-DHPA inhibited the cytopathogenic effects of VSV, vaccinia, and herpes simplex 1 (strain KOS) at a concentration of about 10 to 20 μg/ml, the following congeners of (*S*)-DHPA did not demonstrate antiviral activity at 100 μg/ml (the highest concentration tested): (*S*)-9-(2,3-dihydroxypropyl)hypoxanthine, (*R,S*)-9-(2-hydroxypropyl)adenine, 9-(2-hydroxyethyl)adenine, 9-(2-aminoethyl)adenine, 9-(β-DL-alanyl)adenine, (*S*)-9-(3,4-dihydroxybutyl)adenine, (*R,S*)-9-(3,4-dihydroxybutyl)-adenine, (*R,S*)-*threo*-9-(2,3,4-trihydroxybutyl)adenine, (*R,S*)-9-(3,5-dihydroxypentyl)adenine, (*S*)-1-(2,3-dihydroxypropyl)-thymine, (*R*)-1-(2,3-dihydroxypropyl)thymine, (*S*)-3-(2,3-dihydroxypropyl)thymine, (*S*)-1-(3,4-dihydroxybutyl)uracil, (*R,S*)-1-(3,5-dihydroxypentyl)uracil, (*S*)-1-(2,3-dihydroxypropyl)uracil, 9-(3-hydroxypropyl)adenine, and 2-(9-adeninyl)propane-1,3-diol.

Neither (*S*)-DHPA nor (*R,S*)-DHPA exhibited marked antibacterial or antifungal activity. Tests carried out at the Central Research Institute of Pharmacy and Biochemistry in Prague revealed that (*S*)-DHPA and (*R,S*)-DHPA were not inhibitory to *Streptococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris*, and *Escherichia coli* at concentrations up to 100 μg/ml. For *Mycobacterium tuberculosis, Trichophyton mentagrophytes, Candida albicans*, and *Aspergillus niger* the minimum inhibitory dose was 50 μg/ml.

Unlike ribavirin, which inhibited cellular DNA and RNA synthesis at about the concentrations required for an antiviral effect (*2*), (*S*)-DHPA did not significantly reduce DNA synthesis (monitored by the incorporation of either 2′-[methyl-^{3}H]deoxythymidine or 2′-[2-^{14}C]deoxyuridine) or RNA synthesis (as monitored by [5-^{3}H]uridine incorporation) in HeLa, Vero, and primary rabbit kidney cells, even at concentrations as high as 200 μg/ml. Similarly, (*S*)-DHPA did not inhibit protein synthesis (monitored by [4-^{3}H]leucine incorporation) when applied to primary rabbit kidney cells at a concentration of 400 μg/ml. It did not impair the viability of the uninfected host cells unless extremely high concentrations were employed; for example, to reduce the proliferation of mouse L-929 cells by 50 percent (at 3 days of exponential growth) an (*S*)-DHPA concentration of approximately 600 μg/ml was required, a dose far in excess of that required to inhibit virus replication.

Whether (*S*)-DHPA lacks the undesirable side effects that hamper the usefulness of ribavirin (*4, 5*) is yet to be determined. The mechanism by which it restricts virus replication also remains to be established. Some observations may seem relevant to the mode of action of (*S*)-DHPA. First, it did not inhibit the multiplication of togaviruses and picornaviruses, two virus families that contain a plus strand RNA genome. Second, it was equally effective against herpes simplex 1 (strain KOS) and herpes simplex 2 (strain 333), although under our assay conditions the former induces pyrimidine deoxyribonucleoside kinase activity in primary rabbit kidney and human skin fibroblasts whereas the latter does not do so (*9*).

(*S*)-DHPA is not a substrate for adenine deaminase or adenosine deaminase of either bacterial origin (*E. coli* and *Salmonella typhimurium*) or mammalian origin (rabbit kidney and calf intestine) (*10*). In fact, (*S*)-DHPA strongly inhibits the deamination of both adenosine and ara-A (adenine arabinoside) by adenosine deaminase (from calf intestinal mucosa and primary rabbit kidney cells) (*10*). The latter phenomenon may also explain why (*S*)-DHPA potentiated the inhibitory action of ara-A on the replication of vaccinia and herpes simplex virus, which we observed in our recent experiments.

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Imprinting Behavior: Pituitary-Adrenocortical Modulation of the Approach Response

Abstract. *Plasma corticosterone concentrations in newly hatched ducklings exposed to an imprinting model are inversely related to the strength of approach behavior. Injections of corticosterone before imprinting reduces following, whereas* α^{1-10}*-adrenocorticotropin or antiserum to corticosterone augments following behavior. The sensitive period for imprinting may be regulated by changes in the pituitary-adrenocortical axis.*

In his writings on the development of social behavior in birds, Lorenz (*1*) drew attention to the imprinting process in precocial species. Newly hatched chicks or ducklings will approach and follow the first moving object they see and develop an attachment or preference for this object. The strength of this behavior is important, since it brings and keeps the young bird in contact with a mother figure, thus permitting the learning or imprinting process to occur. I now report that the pituitary-adrenocortical system influences the filial approach and following behavior of imprinting.

Imprinting in young waterfowl and in newly hatched chicks occurs during a "sensitive" period shortly after hatching (*2*). During this early period, the plasma corticosterone concentration in Pekin ducklings increases rapidly (*3*). Because adrenocorticotropin (ACTH) and corticosteroids influence sensory function and learning processes (*4*), I began an investigation of the significance of this hormonal system in the imprinting context.

In these experiments, mallard ducklings were hatched in groups from eggs taken from a resident flock of game-farm mallards. The incubator was dark continuously, and birds were taken individually from the hatcher to the imprinting chamber in small lighttight containers. Ducklings used in the experiment were 15 to 24 hours old. Birds received intraperitoneal injections of either 1 I.U. of $\alpha_p{}^{1-39}$-ACTH (Schering) or 0.1 ml of 0.75 percent saline 20 minutes before testing (group 1); 10 μg of α^{1-24}-ACTH (Organon) or 0.1 ml of $ZnCl_2$ phosphate buffer 30 minutes before testing (group 2); 5 μg of α^{1-10}-ACTH or 0.1 ml of 0.75 percent saline 30 minutes before testing (group 3); 500 ng of corticosterone or 0.1 ml of propylene glycol, 0.75 percent saline, and ethanol (in the proportions 5:9:2) vehicle 15 minutes before testing (group 4); 500 ng of progesterone or 0.1 ml of the 5:9:2 propylene glycol vehicle 15 minutes before testing (group 5); or 0.1 ml of rabbit antiserum to a bovine serum albumin corticosterone conjugate or 0.1 ml of 0.75 percent saline 30 minutes before testing (group 6) (*5*). Each duckling was given the injection, color-marked, and returned to the hatcher until testing. The imprinting testing apparatus has been described (*6*). Briefly, the chamber consisted of a 1 by 5 m sand-covered runway or track along which moved either a blue ball (21-cm diameter) or a red cube (20 cm on side). These models each contained a loudspeaker that emitted the sounds "komm-komm" at fixed intervals; they were suspended from an overhead track by a Plexiglas rod and moved back and forth at a speed of approximately 11 cm/sec. The duckling was placed beside the appropriate model in the dark for 30 seconds before the chamber lights and model voice were activated; 30 seconds later the model began to move. The model moved back and forth in the runway for 10 minutes, and the experimenter recorded (i) the time from the onset of movement of the model until the duckling began to follow it (latency) and (ii) the number of seconds the duckling followed during the 10-minute training (following time). All ducklings were trained with the red cube rather than blue ball except the ducklings that received porcine ACTH and those subsequently used for the corticosterone determination. Ducklings in the latter group, which were exposed for 17 minutes to the blue ball, were decapitated 20 minutes after training; the trunk blood was centrifuged, and plasma was frozen and stored for later assay (*7*). Several trials of each injection experiment were run, each of which was accompanied by control injections of vehicle alone, as described above. Subjects in each trial were newly hatched birds. Because of differences in atmospheric pressure (*8*) and other variables, differences in amount of following are commonly encountered in imprinting studies. Therefore, the data were standardized as group mean variates and depicted as percentage of deviation from control medians. Statistical evaluation was with the Mann-Whitney U test or the product moment correlation coefficient.

The effects of administering various hormones on approach behavior are summarized in Fig. 1. The administration of corticosterone inhibited the approach and following response (*9*). Ducklings waited longer before following the imprinting model and spent less time with the model during the 10-minute imprinting period. Those birds which received antiserum to corticosterone followed more quickly and followed longer during the training period than did controls (*10*). Ducklings receiving α^{1-10}-ACTH, a noncorticotropic (*11*) peptide that contains the first ten amino acids of ACTH, followed more quickly and longer than controls did. This result contrasts with those birds receiving the corticotropic porcine ACTH or synthetic corticotropin, α^{1-24}-ACTH (*11*). Neither of these substances significantly affected latency or following time. Progesterone, as well, had no effect on behavior.

Endogenous corticosterone concentrations are also inversely related to following time (Fig. 2). The data raise the question of whether differences in plasma corticosterone concentrations result from differences in the bird's reaction to the imprinting situation or whether they are the cause of these differences. My experimental manipulations of steroid and ACTH concentrations (Fig. 1) indicate that the hormonal differences cause the behavioral differences.

Data from the injection experiments (Fig. 1) suggest that ACTH and adrenocorticoids reciprocally modulate approach behavior, with the ACTH peptide facilitating the response and corticosterone inhibiting it. The action of corticosterone antiserum may be attributable to a reduction of corticosterone-induced inhibition and, possibly, to an increase in endogenous ACTH concentrations as well, since the hypothalamic-pituitary-adrenal

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