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EFFECTS OF CHIRALITY IN 9-(2-HYDROXY-3-NONYL)ADENINE UPON DEOXYRIBONUCLEIC ACID SYNTHESIS IN HERPES SIMPLEX VIRUS-INFECTED CELLS

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Abstract—The antiherpes activities of *erythro*- and *threo*-9-(2-hydroxy-3-nonyl)adenines (EHNA and THNA) have been determined. All isomers inhibited the replication of herpes simplex virus (HSV) and inhibited DNA synthesis in HSV-infected cells. The two enantiomers of EHNA, (+)-EHNA and (−)-ENHA, displayed equal antiviral activities. This is in contrast to their activities as inhibitors of adenosine deaminase (ADA); (+)-EHNA is a 250-fold more potent inhibitor of ADA than (−)-EHNA [Bessodes *et al. Biochem. Pharmac.* **31**, 879 (1982)]. The antiherpes activity of (+)-THNA was only slightly less than that of the EHNA isomers, whereas (−)-THNA was somewhat less active. The abilities of the four isomeres of EHNA and THNA to inhibit DNA synthesis in HSV-infected cells correlated with their abilities to inhibit virus multiplication. EHNA failed to inhibit HSV DNA polymerase activity in extracts from infected cells. Moreover, addition of EHNA to infected cells at 6 hr post-infection resulted in no inhibition of DNA synthesis. These results are inconsistent with a direct inhibition of macromolecular DNA synthesis by EHNA. Treatment of HSV-infected cells with EHNA produced a 2- to 4-fold decrease in levels of the four DNA precursors, deoxyribonucleoside 5′-triphosphates (dNTPs). This treatment had much less effect on dNTP levels in uninfected cells.

Several inhibitors of adenosine deaminase (ADA) have been developed and studied extensively in recent years. The original goal of these efforts was to develop inhibitors of ADA for use in combination chemotherapy with adenosine analogs. One adenosine analog, 9-β-D-arabinofuranosyladenine (araA, vidarabine), has been approved for topical and systemic treatment of human herpetic disorders, but its efficacy has been minimized by rapid deamination to 9-β-D-arabinofuranosylhypoxanthine (araHx). The combination of araA with inhibitors of ADA has proven successful in experimental systems (reviewed in Ref. 1). In addition, inhibitors of ADA have shown promise in treatment of certain lymphocytic leukemias and as immunosuppresive agents [2]. These latter studies were prompted by the association of ADA deficiency with severe-combined immunodeficiency disease (SCID) in humans [3]. Another, rather surprising, finding was that some inhibitors of ADA display antiviral activity [4, 5].

Two inhibitors of ADA have been studied extensively. The first of these, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA)†, was rationally designed and synthesized by Schaeffer and Schwender [6]. EHNA is a semi-tight binding inhibitor of ADA with a K_i of 2–4 × 10^{-9} M [7]. The second, 2′-deoxycoformycin (DCF, pentostatin), was isolated from cultures of *Streptomyces antibioticus* by researchers at Parke, Davis & Co. [8]. DCF is a tight binding inhibitor of ADA with a K_i in the range of 2.5–15 × 10^{-12} M [7]. Another compound, coformycin, which is different from DCF only in having a ribose rather than a 2′-deoxyribose sugar, is similar to DCF in its activity against ADA [7].

Both EHNA and DCF have been shown to potentiate the cytotoxic activity of adenosine analogs. This was demonstrated first with EHNA by Plunkett and Cohen [9] who showed that EHNA potentiates the activity of araA and of 3′-deoxyadenosine (cordycepin) against cultured cells and against tumor-bearing mice. Similar results were later reported with DCF [10, 11]. Both EHNA and DCF have also been shown to increase the plasma level and half-life of araA in mice [12, 13] and to increase the intracellular concentration of the active metabolite, araATP [14]. The antiviral activity of araA is also potentiated by EHNA [4] or by DCF [15].

In contrast to all of these similarities, EHNA displays antiherpes activity but DCF does not [4]. This antiviral activity is also somewhat selective; a concentration of EHNA (10^{-5} M) that blocks the replication of herpes simplex virus (HSV) by 75% has no effect on cell growth or viability [4]. Although the mechanism of this activity has not been deter-

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† Unless otherwise indicated, EHNA refers to the racemic mixture of two enantiomers which are discussed below.

mined, the inhibition of virus replication correlates with inhibition of viral DNA synthesis [4]. Another inhibitor of ADA which is similar in structure to EHNA, (S)-9-(2,3-dihydroxypropyl)adenine, also has antiviral activity [5], although it has not been determined whether its antiherpes activity is due to inhibition of viral DNA synthesis.

The failure of DCF to block replication of HSV suggests that the antiviral activity of EHNA is due to its action at a site other than ADA. EHNA is known to affect other pathways of purine and pyrimidine metabolism when it is present at high concentrations [16, 17]. Although it is possible that the antiviral activity is due to one of these effects on cellular metabolism, it is more likely that it is directed against a viral component in view of its selective inhibition of HSV DNA synthesis [4].

EHNA is ineffective in treatment of HSV infections in mice [18]. This might have been expected from its failure to completely inhibit virus replication in cultured cells [4]. Nevertheless, we feel that it is important to characterize the mechanism by which it reduces viral DNA synthesis. When this mechanism is understood, we hope to develop more potent inhibitors which block HSV replication but do not inhibit ADA.

Previous studies of EHNA have been performed with a racemic mixture of this compound. Recently, chiral synthesis of these two enantiomers, (+)-*erythro*-9-(2S-hydroxy-3R-nonyl)adenine [(+)-EHNA] and (−)-*erythro*-9-(2R-hydroxy-3S-nonyl)adenine [(−)-EHNA], as well as the two threo enantiomers, (+)-*threo*-9-(2R-hydroxy-3R-nonyl)adenine [(+)-THNA] and (−)-*threo*-9-(2S-hydroxy-3S-nonyl)adenine [(−)-THNA], have been achieved, first by Bastian *et al.* [19] and subsequently by Baker and Hawkins [20]. It was shown that one of these isomers, (+)-EHNA, is much more potent an inhibitor of ADA than the others [21]. In the work presented here, we have examined the antiviral activities of the four stereoisomers. In addition, the effect of EHNA upon DNA metabolism in herpes-infected cells has been explored further.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of HeLa F cells were grown and maintained in Joklik-modified Minimal Essential Medium supplemented with 10% horse serum (both from Grand Island Biological Co., Grand Island, NY) as previously described [4]. Experiments were all done with media containing 10% dialyzed horse serum to minimize interference that might arise from exogenous nucleosides. The Miyama strain of HSV type 1 was grown and maintained as previously described [4]. In all experiments with HSV, HeLa cells in subconfluent monolayers were infected with HSV at an input multiplicity of 10 plaque-forming units (PFU)/cell as previously described [4]. Cell and virus stocks were assayed for mycoplasma contamination by fluorescent staining [22] or by the ratio of uracil and uridine incorporation [23] and were consistently negative.

Materials. Preparation of the stereoisomers of EHNA and THNA has been reported [19]. Racemic (±)-EHNA was provided by H. Schaeffer and G. B. Elion (Wellcome Research Laboratories, Research Triangle Park, NC). Radioactive materials, [*methyl*-^{3}H]thymidine (6.7 Ci/mmole), [*methyl*-^{3}H]dTTP (18.7 Ci/mmole), [5-^{3}H]dCTP (24.8 Ci/mmole), [8-^{3}H]dATP (23.5 Ci/mmole) and [8-^{3}H]dGTP (11.8 Ci/mmole) were all purchased from the New England Nuclear Corp., Boston, MA. Non-radioactive deoxyribonucleosides and deoxyribonucleoside 5′-triphosphates (dNTPs) were obtained from the Sigma Chemical Co., St. Louis, MO. *Escherichia coli* DNA polymerase I was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. All other materials for quantitation of dNTPs were as previously described [24].

Methods. Procedures for measurement of the production of progeny virus from HSV-infected cells and for measurement of DNA synthesis from incorporation of exogeneous supplied [^{3}H]thymidine into trichloroacetic acid-insoluble material are as previously described [4, 22].

Extracts from cells were prepared for analysis of dNTPs by a modification of previous procedures [24]. Medium was removed from cells, which were growing in monolayers in 100 mm Petri plates, and 2.0 ml of ice-cold 0.5 N perchloric acid was added to each plate. Cells were scraped from plates with a rubber policeman and transferred to centrifuge tubes. Petri plates were washed with an additional 1.0 ml of 0.5 N perchloric acid, and the combined cell suspensions and washes were allowed to sit at 4° for 15 min with occasional vortex mixing. These mixtures were then centrifuged at 27,000 *g* for 15 min, and supernatant fractions were removed. Pellets were washed with another 1.0 ml of 0.5 N perchloric acid each, and these washes were combined with the above supernatant fractions and then neutralized by addition of 1.5 N KOH. After precipitates of $KClO_4$ were removed by centrifugation (27,000 *g* for 10 min), precipitates were washed with 0.5 ml water, and then combined washes and supernatant fractions were frozen (−80°) and taken to dryness by lyophilization. Residues were resuspended in 3.0 ml of 60% methanol (−20°) and allowed to sit at −20° for at least 1 hr. These were then centrifuged at 27,000 *g* for 15 min. Pellets were washed with an additional 1.0 ml of 60% methanol, and the washes and supernatant fractions were combined. The methanol was removed by evaporation under a gentle stream of air while holding extracts on ice. Samples were then frozen (−80°) and taken to dryness by lyophilization. Samples were finally resuspended in water, and dNTP determinations were performed as previously described [24]. All values are reported as pmoles dNTP per 10^6 cells. It should be noted that for HSV-infected cells these values do not take into account changes in cell volume that might occur after infection and so do not directly reflect concentrations of dNTPs. All results are representative of two or more experiments.

The HSV DNA polymerase was assayed as described by Weissbach *et al.* [25]. Cells for these experiments were grown in 75 cm^2 tissue culture flasks and infected with HSV at an input multiplicity of 10 PFU/cell. Infected cells were removed from flasks by trypsinization at 6 hr post-infection, col-

lected by centrifugation at 1000 *g* for 5 min, washed twice with phosphate-buffered saline [26], and then resuspended in 10 mM Tris–HCl, pH 7.5, 150 mM KCl, 0.5 mM dithiothreitol and disrupted by sonic oscillation. Protein determinations were as described by Bearden [27].

RESULTS

Inhibition of HSV replication by isomers of EHNA and THNA. It was shown previously that HSV replication is inhibited by EHNA but not by DCF [4]. This suggests that the antiviral activity of EHNA is not due simply to inhibition of ADA. To evaluate this further, we have examined the antiherpes activity of the stereoisomers of EHNA and THNA that were synthesized recently [19]. Structures of these stereoisomers are shown in Fig. 1. One of these, (+)-EHNA, is much more potent as an inhibitor of ADA than the other three isomers. It is 250-fold more active as an inhibitor of this enzyme than (−)-EHNA and is 40–60 times more potent than either of the THNA isomers [21]. Figure 2 shows the effects of these compounds upon multiplication of HSV. Production of progeny virus was blocked equally well by (+)-EHNA and (−)-EHNA, each of which was about equal in activity to the racemic mixture, (±)-EHNA. Both of the THNA enantiomers also inhibited the replication of HSV. The activity of (+)-THNA was only slightly less than that of the EHNA isomers, whereas (−)-THNA was even less active (Fig. 2). The equivalent antiviral activity of the EHNA enantiomers is in sharp contrast to their different potencies as inhibitors of ADA. These data are in agreement with previous results which suggested that the antiherpes activity of EHNA is not due simply to inhibition of ADA.

Inhibition of HSV DNA synthesis by isomers of EHNA and THNA. It was shown previously that (±)-EHNA inhibits DNA synthesis in HSV-infected cells but not in uninfected cells [4]. In those studies it was also shown that this inhibition does not occur

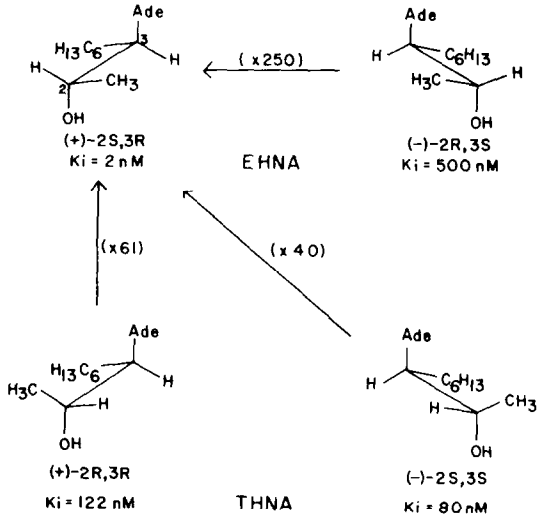

Fig. 1. Structures of the chiral isomers of EHNA and THNA. The inhibition constants were obtained with human erythrocyte ADA as described previously [21].

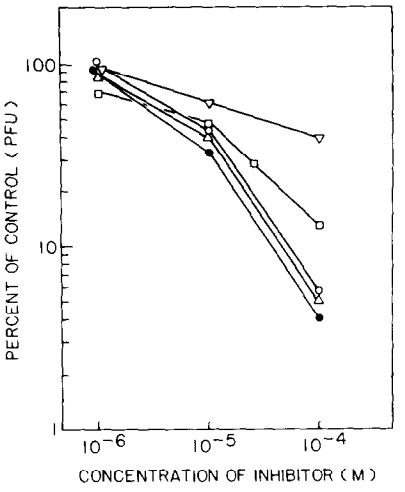

Fig. 2. Effects of the chiral isomers of EHNA and THNA upon multiplication of HSV. Production of progeny virus was determined following infection of HeLa cells by HSV in the presence of the indicated concentrations of racemic (±)-EHNA (●), (+)-EHNA (○), (−)-EHNA (△), (+)-THNA (□), or (−)-THNA (▽). Similar results were obtained in three separate experiments.

until several hours after infection and that its timing coincides with the onset of viral DNA replication. We have examined the isomers of EHNA and THNA to determine whether they block DNA synthesis in a similar way. Results of these experiments are shown in Fig. 3.

Both isomers of EHNA impeded DNA synthesis in HSV-infected cells with a pattern that was nearly

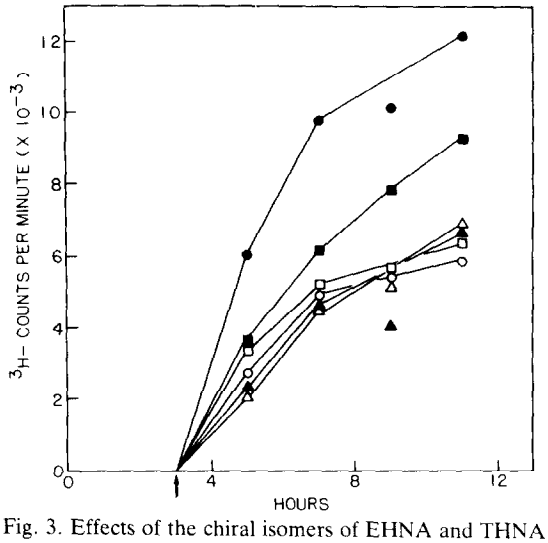

Fig. 3. Effects of the chiral isomers of EHNA and THNA upon DNA synthesis in HSV-infected cells. DNA synthesis was measured by incorporation of [*methyl*-^{3}H]thymidine into trichloroacetic acid-insoluble material (see Methods). The indicated compounds were added at the end of a 1-hr adsorption period (time = 0) and radioactive thymidine was added at t = 3 hr (arrow). These determinations were made of untreated controls (●), or in the presence of 10^{-4} M (±)-EHNA (○), 10^{-4} M (+)-EHNA (△), 10^{-4} M (−)-EHNA (▲), 10^{-4} M (+)-THNA (□), or 10^{-4} M (−)-THNA (■). Similar results were obtained in two separate experiments.

identical to that obtained with (±)-EHNA. The (+)-isomer of THNA also inhibited HSV DNA synthesis in a similar manner. However, (−)-THNA was somewhat less inhibitory to DNA synthesis in HSV-infected cells. These effects on DNA synthesis closely paralleled the abilities of the four isomers to block multiplication of HSV.

Since the two stereoisomers of EHNA showed equal abilities to block the replication of HSV and to inhibit DNA synthesis in HSV-infected cells, we did not worry about chirality in further studies of the mechanism by which EHNA blocks replication of HSV. Accordingly, (±)-EHNA was used in all experiments described below.

Effect of EHNA upon HSV DNA polymerase activity. The HSV-encoded DNA polymerase is active in the presence of high salt (150 mM potassium sulfate) which inhibits the activities of cellular DNA polymerases [25]. This allows specific determination of the activity of the viral enzyme in extracts from HSV-infected cells. We have examined the effect of EHNA upon this activity, and the results are shown in Table 1. The HSV DNA polymerase was not inhibited by 5×10^{-5} M EHNA. This concentration of EHNA inhibits HSV DNA synthesis by 90% [4]. Although we cannot rule out the possibility that a metabolite of EHNA inhibits the HSV DNA polymerase, we do not feel that this is likely. Thus far, no phosphorylated derivatives of EHNA have been detected [28]. Moreover, as described below, EHNA had an effect on the provision of DNA precursors.

In other experiments, we found that treatment of HSV-infected cells with 5×10^{-5} M EHNA failed to block induction of the viral DNA polymerase (data not shown). Moreover, we found that EHNA must be present early in the infection cycle in order to block viral DNA synthesis. Addition of EHNA at 4 hr post-infection caused only a slight inhibition (10%), and addition at 6 hr produced no inhibition of viral DNA synthesis (data not shown). These results are not consistent with a direct inhibition of DNA polymerization by EHNA.

Effect of EHNA upon DNA precursor levels. In further experiments designed to characterize the way in which EHNA blocks HSV DNA synthesis, we examined the effects of EHNA upon levels of deoxyribonucleoside 5′-triphosphates (dNTPs) in HSV-infected cells. As described in Materials and Methods, the techniques previously used for extraction of dNTPs from cells had to be modified to eliminate an activity in HSV-infected cells which degrades dTTP and dCTP to their respective 5′-monophosphates during extraction with 60% methanol. This activity is presumably the deoxypyrimidine triphosphatase reported by Wohlrab and Francke [29] and was eliminated by using 0.5 N perchloric acid in the initial step of extraction (see Methods).

Effects of EHNA upon dNTP levels in HSV-infected and uninfected HeLa cells are shown in Table 2. Levels of all four dNTPs in HSV-infected cells were decreased considerably by 10^{-5} M EHNA and decreased even more by 5×10^{-5} M EHNA. By 6 hr after infection in the presence of 5×10^{-5} M EHNA, levels of dTTP and dGTP had dropped to one-fourth of the levels present in infected cells in the absence of EHNA; levels of dATP and dCTP were decreased to one-half of control levels by this treatment (Table 2). In uninfected cells, the dTTP level was decreased by EHNA in a manner similar to that in HSV-infected cells, but levels of the other three dNTPs were affected much less or not at all.

It has been reported that levels of dTTP, dGTP, and dCTP increase after infection of cells by HSV [30, 31]. Levels of dATP were reported to increase after infection under some conditions [30] and to decrease under others [31]. In the experiments reported here, concentrations of dGTP and dTTP

Table 1. HSV DNA polymerase activities

Extract	Specific activity (units/mg)*	Percent of HSV-infected HeLa
HeLa	<7	<2
HSV-infected HeLa	380	100
HSV-infected HeLa + 5×10^{-5} M (±)-EHNA	388	102

* One unit = 1 nmole/60 min.

Table 2. Effect of EHNA on dNTP pools

	Treatment	dNTP levels (pmoles/10^6 cells) dATP	dTTP	dGTP	dCTP
A.	HSV-infected cells				
	Control	14.0	169.6	34.2	41.2
	10^{-5} M EHNA	11.0	74.1	19.0	36.6
	5×10^{-5} M EHNA	6.4	43.7	9.2	21.2
B.	Uninfected HeLa cells				
	Control	19.2	42.6	8.1	41.0
	10^{-5} M EHNA	20.0	25.2	6.9	48.6
	5×10^{-5} M EHNA	17.3	19.7	11.7	31.5

increased 4-fold after HSV infection, the dCTP level was unchanged (although in some experiments dCTP levels increased 1.5-fold), and the dATP level was decreased. The presence of EHNA (5×10^{-5} M) blocked this expansion of dTTP and dGTP pools (so that their levels remained comparable to those of uninfected cells) and decreased levels of dCTP and dATP to values lower than in uninfected cells (Table 2). It has not been determined whether expansion of one or more dNTP pool is necessary in order to achieve a maximal rate of HSV DNA synthesis. If an increased concentration of one or more precursor is necessary, then EHNA may impede HSV DNA synthesis by limiting the availability of DNA precursors.

DISCUSSION

In the studies reported here, we found that the two enantiomers of EHNA have equal antiherpes activities and equal abilities to block HSV DNA synthesis. This is in contrast to the 250-fold difference in the activities of these two enantiomers as inhibitors of ADA [21]. These data provide further support that the antiviral activity of EHNA is directed against a target other than ADA, which was suggested previously by the failure of DCF to block HSV replication [4]. However, although inhibition of ADA is not by itself sufficient to block replication of HSV, we cannot conclude that inhibition of ADA does not contribute to this effect. The high concentration of EHNA required to block HSV replication is well above the concentration necessary to inhibit ADA. In contrast to the lack of stereoselectivity with respect to antiviral activity of the two isomers of EHNA, the two isomers of THNA are somewhat different in their antiherpes activities.

It was shown previously that EHNA is a selective inhibitor of HSV DNA synthesis [4]. We have shown here that this inhibition of viral DNA synthesis is not due to inhibition of the HSV-encoded DNA polymerase by EHNA (although we cannot exclude the possibility that a metabolite of EHNA will inhibit this enzyme). Furthermore, we have shown that EHNA must be added prior to 6 hr post-infection in order to impede HSV DNA synthesis. This is inconsistent with an inhibition of DNA polymerase by EHNA; these data are also inconsistent with a direct inhibition of macromolecular DNA synthesis by EHNA.

We found that treatment of HSV-infected cells with EHNA caused a substantial decrease in levels of all four dNTPs. The levels of at least some of these dNTPs normally increase after infection of cells by HSV [30, 31], although it has not been determined whether an increased concentration of one or more dNTP is necessary in order to achieve a maximal rate of viral DNA synthesis. It is possible that EHNA impedes HSV DNA synthesis by decreasing the availability of DNA precursors. The failure of EHNA to block DNA synthesis in uninfected cells treated in an identical manner might be explained from the fact that dNTP pools are much less affected in uninfected cells. To evaluate these possibilities further, we need to identify the enzyme(s) affected by EHNA. We do not yet know whether this is a virus-encoded or a cellular enzyme.

EHNA was found previously to be ineffective when used alone for treatment of HSV infections in mice [18]. However, we feel that it should be evaluated further because it seems to be acting at a site that has not yet been employed in viral chemotherapy. We have shown that it does not inhibit the HSV-encoded DNA polymerase. In addition, it is not a substrate for the HSV-encoded thymidine kinase (J. Fyfe, personal communication), and no phosphorylated derivatives of EHNA have been detected [28]. Thus, EHNA is probably not acting via either of the two enzymes that have been exploited in antiherpes chemotherapy. We are hopeful that, once the antiviral activity of EHNA is understood, we can design more potent inhibitors of HSV replication that act upon the same site. Moreover, it would be desirable for these compounds to not inhibit ADA since long term inhibition of this enzyme may impede the immune response [3].

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