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PHARMACOKINETICS OF INHIBITION OF ADENOSINE DEAMINASE BY ERYTHRO-9-(2-HYDROXY-3-NONYL)ADENINE IN CBA MICE

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Abstract—The pharmacokinetics of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) inhibition of adenosine deaminase (ADA) was measured *in vivo* in CBA mice. The *in vivo* assay utilized injection of 10–100 nmoles [2-^{3}H]adenosine and measurement of blood ^3H_2O 20 min later. A single oral dose of EHNA (50 mg/kg) totally inhibited ADA for 4 hr and caused a large increase in conversion of [2-^{3}H]adenosine to [2-^{3}H]ATP. EHNA (3 mg/kg) decreased deamination by 50% for 2–6 hr, depending on the dose of adenosine used. Mice dosed with EHNA (100 mg/kg) once daily for 7 days showed the same ADA recovery rate as mice dosed only once. High single oral doses of EHNA had no effect on blood ATP and GTP pools.

Adenosine deaminase (ADA†; EC 3.5.4.4) catalyzes the deamination of adenosine, 2′-deoxyadenosine and many adenosine analogues. The high level of adenosine deaminase found in blood and other tissues [1] normally prevents the build-up of toxic levels of adenosine or 2′-deoxyadenosine in cells. But adenosine deaminase can also inactivate adenosine analogues such as adenine arabinoside which may be used in the treatment of cancer. To limit this deamination, several ADA inhibitors, including EHNA, were shown to potentiate the anti-tumor effects of adenosine analogues [2–4]. In studies with purified ADA, EHNA was competitive with adenosine and was classified as a semi-tight binding inhibitor [5, 6]. The K_i (EHNA) for human erythrocyte ADA has been reported as 1.6×10^{-9} M [6].

Many cellular effects modulated by elevated adenosine concentrations are further enhanced by EHNA. Lymphocyte-mediated cytolysis of EL4 tumor cells was suppressed by adenosine, and EHNA potentiated this effect [7]. PRPP depletion in lymphoblasts, caused by adenosine, was enhanced by EHNA [8]. Adenosine in combination with EHNA suppressed blastogenesis in phytohemagglutinin-stimulated human lymphocytes [9].

Interest in the combination of EHNA with adenosine analogues in several cellular systems prompted this study to establish pharmacokinetic parameters in the mouse. A rapid method for determining ADA activity in whole animals has been developed. By means of this method, the effectiveness of EHNA as an *in vivo* inhibitor of ADA and the biological half-life of EHNA administered by a variety of routes and schedules have been investigated.

MATERIALS AND METHODS

Animals. CBA mice were obtained from Jackson Laboratories, Bar Harbor, ME. The mice used were from 7 to 11-weeks-old, but the average age was 9 weeks. The average weight was 25 g.

Chemicals. All chemicals used were reagent grade. EHNA·hydrochloride was prepared in these laboratories as described by Schaeffer and Schwender [5]. Activated charcoal (Darco-G-60) was purchased from Matheson, Coleman & Bell (Cincinnati, OH). Nucleosides and bases were obtained from P-L Biochemicals, Inc. (Milwaukee, WI). Panheparin was obtained from Abbott Laboratories (North Chicago, IL).

Radiochemicals. The Amersham Corporation (Arlington Heights, IL) supplied [2-^{3}H]adenosine (sp. act. 24 mCi/μmole), [2-^{3}H]adenine (sp. act. 17 mCi/μmole), and [8-^{14}C]ATP (sp. act. 52 mCi/mmole). Radioactive samples were counted in ACS scintillation solution (Amersham Corp.) and corrected for efficiency against the internal standards [^{3}H]-*n*-hexadecane or [^{14}C]-*n*-hexadecane (Amersham Corp.).

High performance liquid chromatographic separations. Nucleotides were separated on a Partisil 10/SAX column (Whatman Inc., Clifton, NJ), 4.6 mm × 25 cm, with gradient elution between 0.015 M and 1 M KH_2PO_4, pH 3.5, and quantitated as described in detail by Marr *et al.* [10]. Nucleosides and bases were separated on a Partisil 10/ODS column (Whatman Inc.), 4.6 mm × 25 cm, with gradient elution between 0.5 and 50% ethanol [10].

Analysis of adenosine deaminase inhibition in vivo. This method of measuring ADA activity in a whole mouse is based on the high endogenous activity in mice of purine nucleoside phosphorylase (EC

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† Abbreviations: ADA, adenosine deaminase; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; h.p.l.c., high performance liquid chromatography; ara-A, 9-β-D-arabinofuranosyladenine; PCA, perchloric acid; and PRPP, 5′-phosphoribosyl-1-pyrophosphate.

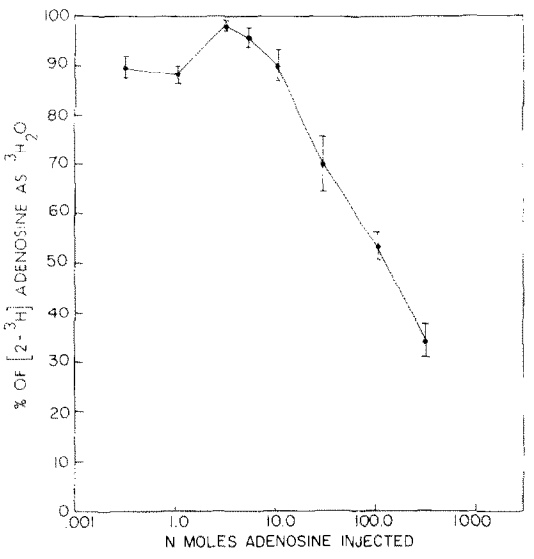

Fig. 1. Deamination of [2-^{3}H]adenosine 20 min after intraperitoneal injection of 10.5 μCi and various concentrations of adenosine. Percent ^3H_2O was determined as described in Materials and Methods.

2.4.2.1) and xanthine oxidase (EC 1.2.3.2), which rapidly and nearly quantitatively convert the product of the ADA reaction, [2-^{3}H]inosine, to xanthine and tritiated water (^3H_2O).

EHNA was dissolved in water and administered directly into the stomach through a feeding needle. At desired times after this initial dose, 10.5 μCi of [2-^{3}H]adenosine in 0.2 ml (sp. act 1.0 μCi/nmole or 0.1 μCi/nmole) was injected intraperitoneally. Each mouse was decapitated after 20 min and allowed to bleed into a beaker containing approximately 5 units of Panheparin. Two-tenths ml of blood was added to 2 ml of ice-cold 0.5 N perchloric acid, and 0.1 ml of [^{14}C]ATP (10,000 dpm) was added as a recovery marker. After centrifugation, 0.1 ml of clear supernatant fluid was counted by a double label procedure to determine total carbon-14 and tritium radioactivity in the blood. A 0.5-ml aliquot of the supernatant fluid was mixed with 10 mg of Darco charcoal and vortexed occasionally for 30 min to remove all purine-associated radioactivity. Greater than 99.7% of the [^{14}C]ATP was adsorbed by the charcoal in this step, thus confirming the complete removal of all purines. After centrifugation to remove the charcoal, 0.1 ml of the charcoal-treated supernatant fluid was counted. The remainder of the PCA extract was neutralized and frozen until analysis by high performance liquid chromatography. From the radioactivity in the blood extract before and after charcoal, the percentage of radioactivity in blood at that time due to ^3H_2O and to [^{3}H]adenine compounds, as well as the total nmoles of each, was calculated.

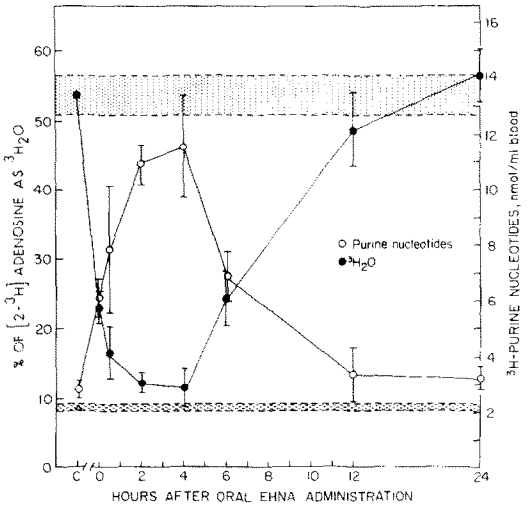

Fig. 2. Percent ^3H_2O and nmoles of [^{3}H]purine nucleotides versus time after an oral dose of 50 mg EHNA/kg. Adenosine (108 nmoles, 10.5 μCi) was injected at each time point and the mice were killed 20 min later. Each point shows the mean ± S.E. of five mice. The upper shaded band represents the mean ± S.E. of uninhibited controls. The lower shaded band represents the mean ± S.E. of an adenine control, i.e. total ADA inhibition.

RESULTS

Optimal conditions for the assay of ADA in vivo. The dose of [2-^{3}H]adenosine selected for the *in vivo* assay needed to be low enough for rapid deamination by uninhibited ADA but high enough to be well above the K_m of adenosine deaminase. It also needed to be well above normal levels of endogenous adenosine to minimize specific activity dilution of injected adenosine. Adenosine levels in human plasma have been reported as 0.07 nmoles/ml in normal subjects and 0.93 nmoles/ml in a patient with severe combined immunodeficiency [11]. Figure 1 shows the percentage of [2-^{3}H]adenosine converted to ^3H_2O in blood after 20 min over a thousand-fold concentration range of [2-^{3}H]adenosine. Twenty min was the length of time required for the complete deamination of up to 10 nmoles of [2-^{3}H]adenosine. At this concentration and time, [2-^{3}H]adenosine was metabolized to more than 90% ^3H_2O with the remainder present primarily as ATP in the red cells. Larger doses of adenosine were incompletely deaminated by 20 min. After EHNA administration to mice, the decreased formation in blood of tritiated water from [2-^{3}H]adenosine served as a measure of ADA inhibition.

[2-^{3}H]Adenine, which is not subject to direct deamination in mammalian cells, was converted only 10–12% to ^3H_2O. In the figures this is referred to as the adenine control and represents the ^3H_2O derived from pathways not involving ADA. EHNA had no effect on the metabolism of [2-^{3}H]adenine to ^3H_2O.

Two types of experiments were performed based on the amount of [2-^{3}H]adenosine administered to mice. The experiments utilizing 10 nmoles [2-^{3}H]adenosine would give maximum sensitivity in inhibition measurement and also would more closely mimic physiological levels of adenosine and 2'-deoxyadenosine. This concentration would also have a minimal effect on normal physiological processes.

A higher level of adenosine, 100 nmoles, was used

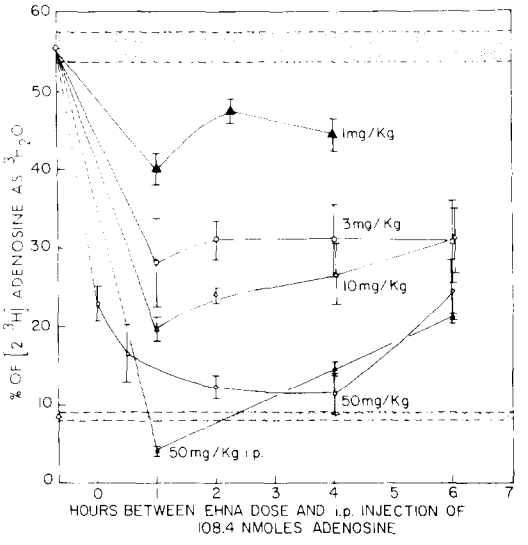

Fig. 3. ADA inhibition with various oral and intraperitoneal doses of EHNA and intraperitoneal injection of 108.4 nmoles [2-^{3}H]adenosine (10.5 μCi). Doses of EHNA were oral unless specified as i.p. Each point shows the mean ± S.E. of five mice.

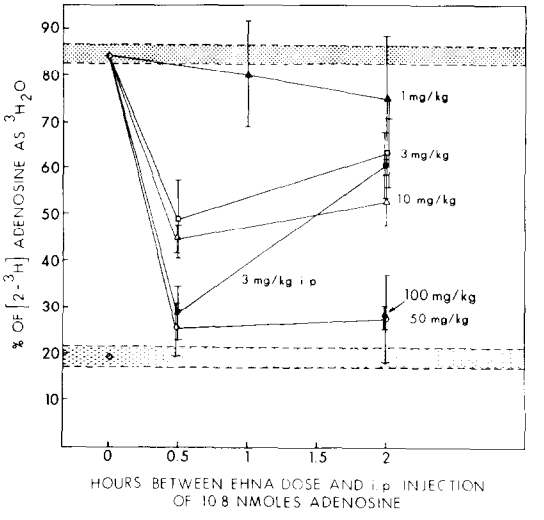

Fig. 4. ADA inhibition with various oral and intraperitoneal doses of EHNA and intraperitoneal injection of 10.8 nmoles [2-^{3}H]adenosine (10.5 μCi). Doses of EHNA were oral unless specified as i.p. Each point shows the mean ± S.E. of 5 mice. One hundred mg/kg is represented by only a 2-hr point.

in other experiments to saturate ADA more completely for the full 20-min assay period and minimize substrate concentration changes. The experiments with high level adenosine also are more comparable to high levels of adenosine analogs used in chemotheraphy.

Effect of single oral doses of EHNA on ADA activity in vivo. In most of these experiments EHNA was administered orally since it is orally effective and this route might have clinical application. When EHNA was administered at 50 mg/kg, a large increase in conversion of [2-^{3}H]adenosine to ATP and a concomitant reduction in the amount of ^3H_2O in the blood were observed (Fig. 2). The time course of ADA inhibition in CBA/J mice *in vivo* at different EHNA concentrations was also investigated, with 108 nmoles of [2-^{3}H]adenosine (Fig. 3) and 10.8 nmoles [2-^{3}H]adenosine (Fig. 4) injected.

These studies show that a single oral dose of EHNA (50 mg/kg) was able to maintain total inhibition of ADA for 4 hr. This was true even when 10 nmoles of [2-^{3}H]adenosine was used. EHNA was rapidly absorbed into the bloodstream. When [2-^{3}H]adenosine was injected immediately after dosing with EHNA, strong inhibition was apparent in the blood sample 20 min later (Fig. 3). EHNA, given intraperitoneally, resulted in greater inhibition at early time points than a comparable oral dose. However, after sufficient time for absorption of the oral dose, inhibition levels were nearly the same for both routes of administration.

The apparent recovery from ADA inhibition was more rapid with low adenosine concentrations than with high. A large fraction of the low dose of [2-^{3}H]adenosine could be deaminated by small amounts of ADA that remained uninhibited or recovered from inhibition. Specific activity dilution may also be a factor since adenosine injected i.p. would enter the bloodstream gradually. Because of these factors, a larger standard error at each time point was seen with 10 nmoles adenosine than with 100 nmoles (Figs. 3 and 4). For example, at 2 hr after the EHNA doses, the average standard error of the four concentrations was 1.7 with 100 nmoles adenosine injected and 9.0 with 10 nmoles adenosine.

Because of this rapid apparent recovery, low levels of adenosine required more EHNA to prevent deamination for a given period of time than high concentrations. Figure 3 shows that, even after 6 hr 3 mg EHNA/kg allowed only half of 100 nmoles adenosine to be deaminated to ^3H_2O. In contrast, 3 mg EHNA/kg was effective for less than 2 hr in maintaining this same percent deamination of 10 nmoles adenosine (Fig. 4).

An attempt was made to extend ADA inhibition by subcutaneous administration of the drug. EHNA at 250 mg/kg in water, subcutaneously, gave less inhibition at early times than 100 mg/kg oral doses, but neither was inhibitory at 24 hr. EHNA, 100 mg/kg in 5% polyvinylpyrrolidone, subcutaneously, had no inhibiting effect on ADA at 24 hr.

Effects of multiple oral doses of EHNA on ADA activity in vivo. In an attempt to prolong the period of total ADA inhibition, multiple doses of EHNA were given, with the second dose given during the average inhibitory period of the first dose (Table 1). With a single dose of EHNA at these concentrations, ADA activity would have returned to control levels in most of the mice after 12 hr. Twenty-four hours after the beginning of double dosing, the average percent ^3H_2O was lower than with single doses, due to strong inhibition remaining in some animals. However, other animals within the same group had

Table 1. Repetitive oral doses of EHNA and percent ^3H_2O produced 24 hr after the initial dose*

EHNA (mg/kg)	Doses/ day	Dose time (hr) 1st	Dose time (hr) 2nd	[2-^{3}H]Adenosine time (hr)	N†	% ^3H_2O ± S.E.
Control					13	84.5 ± 1.9
50	2	0	4	24	5	65.9 ± 8.4
100	2	0	4	24	13	53.9 ± 8.8
100	2	0	12	24	4	78.1 ± 5.9
100	2 × 4 days	0	6	96‡	9	87.3 ± 2.4

* CBA/J mice were given oral doses of EHNA twice daily and then injected with 10.8 nmoles [2-^{3}H]adenosine (10.5 μCi) 24 hr after the first dose. Percent ^3H_2O was determined as described in Materials and Methods.

† Number of mice.

‡ The sample was taken 24 hr after the first dose on day 4.

regained normal ADA activity. This probably reflects slight individual differences in ADA levels as well as variation of EHNA metabolites in tissue and differences in metabolite excretion. Even two doses of 100 mg/kg 4 hr apart allowed normal ADA activity in five out of thirteen mice 24 hr later. A second dose of 100 mg/kg 12 hr after the first acted as a separate single treatment and normal ADA activity was found at 24 hr. Repetitive multiple dosing would be necessary to obtain prolonged maximal inhibition of ADA with EHNA. Also, each dose should be well within the average inhibitory period of the preceding dose. There were no cumulative effects on ADA caused by multiple doses of EHNA during 24 hr.

To determine whether regular exposure to EHNA would cause ADA to become more or less sensitive to inhibition, the rate of recovery of ADA activity was measured after a single dose of EHNA. This was compared to the rate of recovery after 7 consecutive days of dosing with EHNA. Mice dosed once a day for 1 week with 100 mg EHNA/kg had the same rate of recovery from inhibition between 6 and 16 hr as another group receiving only a single dose. This normal rate of recovery within 24 hr was also seen after two doses per day for 4 days (Table 1).

Nucleotide pools in mouse erythrocytes after oral administration of EHNA. ATP and GTP pools in erythrocytes taken from mice at various times after the dose of EHNA were analyzed by h.p.l.c. chromatography. The results of two experiments in Table 2 show that although strong ADA inhibition had occurred, as judged from the decreased percent ^3H_2O, the actual pool size of both ATP and GTP was not significantly altered from normal levels. The trace incorporation of [2-^{3}H]adenosine was too small to affect ATP levels itself, i.e. about 1/1000th of the ATP in the case of the largest incorporation was due to ^{3}H-labeled adenosine.

DISCUSSION

The pharmacokinetics of ADA inhibition by EHNA in mice appears to be consistent with *in vitro* enzyme kinetics for the interaction of EHNA and

Table 2. Determination of nucleotide pools in mouse blood after a single oral dose of EHNA.

EHNA (mg/kg)	Hours after dosing (before [2-^{3}H]adenosine)	N*	% ^3H_2O	ATP (nmoles/ml blood ± S.E.)	GTP (nmoles/ml blood ± S.E)
(A)† Control		5	54	597 ± 30	137 ± 6
50	2	5	12	579 ± 24	107 ± 15
50	12	5	48	527 ± 23	119 ± 2
(B)‡ Control		5	80	608 ± 22	143 ± 5
100	2	6	28	574 ± 37	144 ± 3
100	12	5	24	606 ± 91	123 ± 7

* Number of mice in each group.

† An oral dose of 50 mg EHNA/kg was given to each mouse. After waiting 2 or 12 hr, a "pulse" of [2-^{3}H]adenosine (108 nmoles, 10 μCi) was injected i.p.; 20 min later, the animal was killed. Control animals were dosed with water, and then given a 20 min "pulse" of adenosine. Method of killing and extraction are described in Materials and Methods and analysis was by h.p.l.c.

‡ An oral dose of 100 mg EHNA/kg was given to each mouse. After waiting 2 or 12 hr, a "pulse" of [2-^{3}H]adenosine (10.8 nmoles, 10 μCi) was injected i.p.; 20 min later, the animal was killed. Control animals were dosed with water, and then given a 20-min "pulse" of adenosine. Method of killing and extraction are described in Materials and Methods and analysis was by h.p.l.c.

ADA. The reasons for the relatively short duration of action of EHNA in the mouse are several-fold. EHNA is a reversible inhibitor of ADA and thus the *in vivo* inhibition of ADA is dependent upon plasma and tissue concentrations of EHNA. These concentrations decrease with time as EHNA is metabolized to several hydroxylated derivatives and excreted [12]. Several of these derivatives also inhibit ADA, but none is as effective as EHNA [12].

The average survival time of $BD2F_1$ mice bearing Ehrlich ascites tumor has been shown to be increased by the combination of ara-A (50 mg/kg) and EHNA (3 mg/kg) when compared with ara-A alone [2]. This large dose of ara-A is much larger than the test dose of [2-^{3}H]adenosine used in these studies. As demonstrated in Fig. 3, deamination of ara-A would probably be reduced 50% for 6 hr by 3 mg/kg, although therapeutically effective levels would be prolonged even longer.

Any use of EHNA with mice must take into account the large variations between individual mice in the effects of EHNA, particularly during recovery from the point of maximum inhibition. These differences are probably due to individual differences both in ADA or serum adenosine levels and in breakdown of EHNA to its less inhibitory metabolites. Animals within a group that exhibited large differences in percent ^3H_2O showed a direct correlation between the percent ^3H_2O and the amount of ^{3}H-nucleotides formed. These differences thus reflect real ADA inhibition and not artifacts in measurement. Altering the amount of food in the digestive tract at the time of oral dosing or timing of the dose within the diurnal activity cycle did not affect the magnitude of these variations. These variations appeared greatest when physiologically low concentrations of adenosine were used. The concentration of adenosine or adenosine analog given in an *in vivo* experiment must determine the amount of EHNA used to minimize deamination for the desired time.

Mice dosed with EHNA, 100 mg/kg, once each day for 7 days, were compared with mice given a single dose. Both groups showed the same rate of recovery of ADA activity. This would indicate no enzyme induction by EHNA or altered dissociation constant for EHNA·ADA complex. Infusion of EHNA into mice for 5 days has been reported to result in a lower K_i for the inhibitor [1]. The test reported here would not have detected such a change in K_i.

EHNA had no effect on pools of ATP and GTP in the blood of mice receiving high single oral doses (Table 2). There was increased incorporation of the tracer dose of [2-^{3}H]adenosine into ATP during the inhibitory period, due to increased availability of adenosine for nucleotide formation through adenosine kinase. The effect of closely spaced multiple doses of EHNA on the dATP pool observed previously in blood and thymus [13] is probably the result of sparing of 2′-deoxyadenosine by constant ADA inhibition. A similar increased pool of dATP has been observed in mice treated with 2′-deoxycoformycin once every 24 hr [13] and in the blood of patients with adenosine deaminase deficiency associated with severe combined immunodeficiency disease [14–16]. Elevated levels of deoxyadenosine have been shown to inhibit lymphocyte transformation and be cytotoxic *in vitro* when ADA is inhibited [17]. The accumulation of dATP caused by deoxycoformycin or EHNA was more pronounced with deoxycoformycin, which shows a much longer duration of ADA inhibition than EHNA [6].

EHNA is not only an effective inhibitor of ADA, but has pharmacokinetic properties that would be advantageous in a clinical setting where short-term inhibition of ADA is required, e.g. with concomitant administration of ara-A or other cytotoxic adenosine analogues. *In vivo*, EHNA has a short duration of action on ADA, giving ready control of inhibition of the enzyme. Moreover, normal ribonucleotide pools were not altered after large daily doses of EHNA.

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