A Novel Mechanism-based Inhibitor (6′-Bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine) That Covalently Modifies Human Placental S-Adenosylhomocysteine Hydrolase*

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Most inhibitors of S-adenosylhomocysteine (AdoHcy) hydrolase function as substrates for the “3′-oxidative activity” of the enzyme and convert the enzyme from its active form (NAD+) to its inactive form (NADH). In this study, we describe the effects of a mechanism-based inhibitor, 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine (BDDFHA), which functions as a substrate for the “6′-hydrolytic activity” of the enzyme with subsequent formation of a covalent linkage with the enzyme. Incubation of human placental AdoHcy hydrolase with BDDFHA results in a maximum inactivation of 83% with the remaining enzyme activity exhibiting one-third of the kcat value of the native enzyme. This partial inactivation is concomitant with the release of both Br− and F− ions and the formation of adenine (Ade). The enzyme can be covalently labeled with [8-3H]BDDFHA, resulting in a stoichiometry of 2 mol of BDDFHA/mol of the tetrameric enzyme. The 3H-labeled enzyme retains its original NAD+/NADH content. Tryptic digestion and subsequent protein sequencing of the [8-3H]BDDFHA-labeled enzyme revealed that Arg196 is the residue that is associated with the radiolabeled inhibitor. The partition ratio of the Ade formation (nonlethal event) to covalent acylation (lethal event) is approximately 1:1. From these experimental results, a possible mechanism by which BDDFHA inactivates AdoHcy hydrolase is proposed: enzyme-mediated water addition at the C-6′ position of BDDFHA followed by elimination of Br− ion results in the formation of homoado-6′-carboxaldehyde (HACF). HACF then partitions in two ways: (a) attack by a proximal nucleophile to form an amide bond after expulsion of F− ion (lethal event) or (b) depurination to form Ade and hexose-derived 6-carboxyl fluoride (HDCF), which is further hydrolyzed to hexose-derived 6-carboxylic acid (HDCA) and F− ion (nonlethal event).

S-Adenosylhomocysteine (AdoHcy)3 hydrolyase (EC 3.3.1.1) catalyzes the reversible hydrolysis of AdoHcy to adenine (Ado) and L-homocysteine (Hcy) (1). Because of its important role in regulating biological methylation reactions (2), AdoHcy hydrolase has been selected as a target for the design of antiviral (3–6), antiparasitic (7), antiarthritic (8), immunosuppressive (9), and antitumor agents (10).

Palmer and Abeles (11) elucidated the mechanism by which AdoHcy hydrolase catalyzes the conversion of AdoHcy to Ado and Hcy. The first step involves oxidation of the 3′-hydroxyl group (3′-oxidative activity) of AdoHcy by the enzyme-bound NAD+ (E-NAD+) to form E-NADH and 3′-keto-AdoHcy, which undergoes β-elimination of Hcy to form 3′-keto-4′,5′-didehydro-5′-deoxyAdo. Michael-type addition of water to this tightly bound intermediate (hydrolytic activity) affords 3′-keto-Ado, which is reduced by E-NADH to yield Ado and E-NAD+.

In recent years, significant efforts have been made in designing potent and selective inhibitors of AdoHcy hydrolase (12–18). Most inhibitors of AdoHcy hydrolase are Ado analogs, which inhibit the enzyme by serving as substrates for the 3′-oxidative activity of the enzyme and converting it from the active form (NAD+) to the inactive form (NADH) (6). This type of inhibitor of AdoHcy hydrolase has been defined as a type I mechanism-based inhibitor (5). McCarthy et al. (15, 19) designed and synthesized (Z)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (ZDDFA), an analog of a tightly bound enzyme reaction intermediate, as a potential type II mechanism-based inhibitor. Type II mechanism-based inhibitors are defined as inhibitors that are activated by the enzyme and irreversibly inactivate the enzyme through covalent modification (5). Yuan et al. (20) have shown that ZDDFA is not a type II mechanism-based inhibitor but is a “pro-inhibitor” that is converted into Ado-5′-carboxaldehyde. Enzyme inactivation by ZDDFA does not involve covalent modification; instead, ZDDFA is converted into Ado-5′-carboxaldehyde by the enzyme’s hydrolytic activity, which then inactivates the enzyme by the type I mechanism.

In 1991, Parry et al. (21) showed that an acetylenic analog of adenine (9-(5′,6′-dideoxy-β-D-ribo-hex-5′-ynofuranosyl)adenine) was a type II inhibitor of AdoHcy hydrolase. This acetylenic analog inactivated the enzyme by first serving as a substrate for the 3′-oxidative activity (converting NAD+ to NADH) yielding the 3′-keto acetylenic analog. This tightly bound in

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The abbreviations used are: AdoHcy, S-adenosyl-L-homocysteine; Ado, adenosine; Hcy, homocysteine; Ad, adenosine; EDDFHA, (E)-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine; BDDFHA, 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine; ZDDFA, (Z)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine; HACF, homoadenosine 6′-carboxyl fluoride; HDCA, hexose-derived 6-carboxylic acid; E-NAD+, enzyme-bound NAD+; E-NADH, enzyme-bound NADH; DTNB, 5′,5′-dithiobis(2-nitrobenzoic acid); HPLC, high performance liquid chromatography.
Scheme 1. Kinetic model by which BDDFHA inactivates AdoHcy hydrolase.

Experimental procedures

Materials—AdoHcy, Ado, Hcy, and calf intestinal Ado deaminase (EC 3.5.4.4) were purchased from Sigma. Standard F– and Br– ions were purchased from P. J. Cobert Association, Inc. (St. Louis, MO). Recombinant human placental AdoHcy hydrolase was overexpressed and purified as described previously (22). BDDFHA was synthesized as described.7 and was [8–3H]-labeled by NEN Life Science Products.

Assay of AdoHcy Hydrolase Activity—AdoHcy hydrolase activity was assayed in both the synthetic and hydrolytic directions. In the synthetic direction, the rate of formation of Ado from AdoHcy and Hcy was the product of enzyme activity measured. The enzyme was incubated with 0.2 mm Ado and 5 mm Hcy in 500 μl of 50 mm potassium phosphate buffer, pH 7.2, containing 1 mM EDTA (buffer A) at 37 °C for 5 min. The reaction was terminated by the addition of 25 μl of 5 N HClO4. After the reaction mixture was kept in ice water for 15 min, the clear supernatant was collected and analyzed for AdoHcy by HPLC using a C18 reverse-phase column (Econosphere C18, 5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL) as described earlier (20). In the hydrolytic direction, the assay was performed spectrophotometrically by measuring the rate of the product (Hcy) formed by reaction with DTNB as reported previously (24). To 800 μl of the enzyme solution containing 4.7 μg of AdoHcy hydrolase and 4 units of Ado deaminase in buffer A was added 200 μl of AdoHcy (500 μm) containing 250 μM DTNB in buffer A. The reaction mixture was maintained at 37 °C for 2 min, and the reaction was monitored at 412 nm continuously using an Hewlett-Packard 8432 diode array UV spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). One unit of the enzyme activity is defined as the amount of enzyme that can synthesize or hydrolyze 1 pmol of AdoHcy/min. The protein concentration was determined by the method of Bradford (25), using bovine serum albumin as a standard.

Inactivation of AdoHcy Hydrolase by BDDFHA—Time-dependent inactivation of AdoHcy hydrolase by BDDFHA was measured by incubating various concentrations of the inhibitor (2.5–20 μM) with 20 nm AdoHcy hydrolase in buffer A at 37 °C for different times (0–20 min). The activity remaining was determined in the synthetic direction as described above. Pseudo-first-order rate constants (k_on) were obtained by plotting the logarithm of the remaining activity against time, and the K and k_on values were obtained by the Kitz and Wilson method (26).

Based on the inactivation mechanism shown in Scheme 1, the K and k_inact are expressed in Equations 1 and 2.

\[ K = \frac{k_1}{k_2} \]  
\[ k_{\text{inact}} = k_{\text{de}} (k_2 + k_3 + k_1) \]

Because k_1 and k_2 can be estimated individually as described below, k_4 can be solved according to equation (2). Time-dependent inactivation of the enzyme was also examined in the presence of the substrate Ado (100 μM) by incubation of AdoHcy hydrolase (4 μM) with 100 μM of BDDFHA for various times.

Determination of F– and Br– Ions Released from BDDFHA—F– and Br– ions released from the reaction of AdoHcy hydrolase and BDDFHA were determined by ion-exchange chromatography. The enzyme (28 μM) was incubated with 300 μM BDDFHA in buffer A at 37 °C for various times (0, 5, 10, 20, 30, and 60 min). At each time point, an aliquot of the reaction mixture containing 900 μg of protein (500 μl) was withdrawn and added to a test tube containing 1.5 ml of 97% ethanol. The denatured protein was removed by filtration through an Amicon Centricon-3 microconcentrator (3000 M cutoff), and the filtrate and washing (1 ml) were combined, lyophilized, and redisolvated in 1 ml of water, 30 μl of which was injected onto an ion-exchange column (Anion/R, 250 × 4.1 mm, 10 μM, Alltech) in an HPLC system equipped with a conductivity detector (690 Ion Chromatograph, Omega Metrohm, Ltd., Herisau, Switzerland) for halide ion analysis. F– and Br– ions were eluted isocratically with 1.5 mm μ-hydroxybenzoic acid, 2% methanol (by volume) adjusted by LiOH. The flow rate was 2.0 ml/min. Control experiments were performed using bovine serum albumin and authentic halide ions to assess the recovery from the above procedure. Recoveries routinely were 69–72% in the control experiments. Quantitation of halide ions was achieved by comparison of the ion peak areas with that of known quantities of authentic ions using a standard curve. The first-order rate constants for the release of Br– ion (equal to k_2) and F– ion (approximately k_3 + k_4) were obtained by fitting the data to an exponential function.

Determination of Ado Formation—AdoHcy hydrolase and BDDFHA were incubated in buffer A under the same conditions as described for F– and Br– ion determination, except that at each time point the reaction was stopped by addition of 10 μl of 5 N HClO4. The precipitate was removed by centrifugation, and the supernatant was used for Ado analysis by HPLC using a reverse-phase C18 column as described previously (22). The peak assigned to Ado was confirmed by coinjection with authentic Ado on the HPLC system as well as by mass spectral analysis using chemical ionization with ammonia as the reagent gas. The first-order rate constant (k_5) was obtained by fitting the data to an exponential function.

Determination of E-NAD+ and E-NADH—Quantitation of E-NAD+ and E-NADH was performed using a fluorescence technique (20). The conversion process of the E-NAD+ to E-NADH induced by inhibitors was monitored by UV spectroscopy as described previously (22). The E-NADH (2 mg) in 0.8 ml of buffer A was incubated with 500 μM BDDFHA or 200 μM ZDDFA at 25 °C for 30 min. The inhibitor-induced NADH formation was monitored at 340 nm using an Hewlett-Packard 8452 diode array UV spectrophotometer.

Stoichiometries of Binding and Covalent Labeling—The stoichiometry of binding of BDDFHA to AdoHcy hydrolase was determined by incubating the enzyme with [8–3H]BDDFHA. The enzyme (0.5 mg) in 250 μl of buffer A was incubated with 500 μM [8–3H]BDDFHA (187 μCi/μmol) at 37 °C for 2 h. The reaction mixture was then passed through a Sephadex G-25 column to remove unbound inhibitor. The protein concentration, 3H radioactivity, and remaining enzyme activity in the filtrate were determined, and the ratio of bound [8–3H]BDDFHA was determined by liquid scintillation counting. Peptide peaks containing significant radioactivity were collected and concentrated by Speed-Vac and rechromatographed on the same column using the initial conditions of 2% solvent II (90% acetonitrile, 9.6 mm, Alltech, Deerfield, IL) as described earlier (20).

The first-order rate constants for the release of Br– ion (equal to k_2) and F– ion (approximately k_3 + k_4) were obtained by fitting the data to an exponential function.

Isolation and Identification of [8–3H]BDDFHA-labeled Peptides—Enzyme (2 mg) was dissolved in 0.8 ml of buffer A and incubated with 1 mg of [8–3H]BDDFHA (189 μCi/μmol) at 37 °C for 3 h. The reaction mixture was then gel-filtered through a Sephadex G-25 spin column to remove the free [8–3H]BDDFHA. The enzyme-inhibitor complex was denatured with 8 μl urea and gel-filtered again through the spin column to remove noncovalently bound [8–3H]BDDFHA. The radioactivity and the protein content in the filtrate were used to calculate the stoichiometry of the covalent labeling.

2 S. F. Wnuk, Y. Mao, C. S. Yuan, R. T. Borchardt, G. Andrei, J. Balsarini, E. De Clercq, and M. J. Robins, submitted for publication.
incubation of the enzyme with a large molar excess of [8-3H]-BDDFHA, and removal of the free [8-3H]BDDFHA by gel filtration, the stoichiometry of binding of BDDFHA to the enzyme was approximately 1 mol of BDDFHA/mol of the enzyme subunit as shown in Table I. However, after denaturation of the enzyme-inhibitor complex with urea and removal of the released [8-3H]BDDFHA by gel filtration, the stoichiometry of covalent incorporation of [8-3H]BDDFHA to the enzyme was approximately 0.5 mol of BDDFHA/mol of the enzyme subunit (Table I). When the enzyme was incubated with unlabeled BDDFHA first, and the enzyme-inhibitor complex from gel filtration was then further incubated with [8-3H]BDDFHA, the noncovalently bound BDDFHA (~0.5 mol/ enzyme subunit) was replaced by [8-3H]-labeled BDDFHA, and the remaining enzyme activity was unchanged (~20%). However, when the unlabeled BDDFHA-enzyme complex was further incubated with [8-3H]ZDDFA, a type I mechanism-based inhibitor, the noncovalently bound BDDFHA was replaced by ZDDFA, and the enzyme was completely inactivated. This replacement of noncovalently bound BDDFHA was also observed with the substrate Ado, which prevents further replacement by [8-3H]BDDFHA (Table I), indicating that the enzyme binds Ado in the same vacancy as it binds BDDFHA.

Release of Halide Ions from BDDFHA—Incubation of AdoHcy hydrolase with BDDFHA resulted in the release of both Br⁻ and F⁻ ions. As shown in Fig. 2, when a large molar excess of BDDFHA was incubated with AdoHcy hydrolase, Br⁻ ion (retention time of 6.78 min) and F⁻ ion (retention time of 2.60 min) were observed on the ion-exchange chromatogram, with maxima of about 1.1 mol of Br⁻ ion and 0.9 mol of F⁻ ion/mol of inactivated enzyme subunit. The rate of Br⁻ ion formation (k₂) was faster than that of F⁻ ion formation (assumed approximately equal to k₃ + k₄). The first-order rate constant for Br⁻ ion release was estimated to be 0.27 min⁻¹ (Fig. 2, inset), and the rate for F⁻ ion formation was 0.098 min⁻¹, indicating that Br⁻ ion was released first followed by F⁻ ion.

Formation of Ade—Incubation of AdoHcy hydrolase with BDDFHA resulted in the formation of Ade as shown in Fig. 3. Two main peaks appeared on the HPLC chromatogram. The peak with retention time of 14.84 min was unreacted BDDFHA, and the peak with retention time of 9.69 min was Ade as determined by its same retention time as authentic Ade upon coinjection on HPLC. Its chemical ionization mass spectrometric analysis gave an ion (M⁻) at m/z 136, which is consistent with the mass of Ade. The first-order rate constant of Ade release (k₅) from BDDFHA was estimated to be 0.056 min⁻¹, which is slower than the rates of both Br⁻ and F⁻ ion release. The maximal Ade formation was approximately 0.6

![Figure 1](image.png)

**FIG. 1.** Time-dependent inactivation of AdoHcy hydrolase with BDDFHA in the absence and presence of Ado. AdoHcy hydrolase (4 µM) was incubated with a large molar excess of BDDFHA (100 µM), time-dependent loss of activity was observed as shown in Fig. 1. However, the enzyme was not completely inactivated, and the maximal inactivation was 83% of the original enzyme activity. The enzyme was substantially protected from inactivation by BDDFHA by inclusion of the substrate Ado at a concentration equal to the concentration of BDDFHA (100 µM) (Fig. 1). Kinetic analysis using the Kitz and Wilson method gave a Kᵦ value of 3.9 µM, and kᵦmax value of 0.04 min⁻¹ for BDDFHA inactivation of human AdoHcy hydrolase. The enzyme inactivation by BDDFHA was irreversible, i.e., the activity of the inactivated enzyme could not be recovered by dialysis or gel filtration.

**Stoichiometry of Binding and Covalent Modification—**After

| Reactions | Activity remaining | Total binding | Covalent labeling |
|-----------|--------------------|---------------|------------------|
| E + [8-3H]BDDFHA | 20.2 ± 2.2 | 0.83 ± 0.08 | 0.49 ± 0.05 |
| E-BDDFHA + [8-3H]BDDFHA | 19.3 ± 1.8 | 0.44 ± 0.05 | 0.43 ± 0.01 |
| E-BDDFHA + [8-3H]ZDDFA | 0.05 ± 0.06 | 0.05 ± 0.01 |
| E-BDDFHA + Ado + [8-3H]BDDFHA | 20.8 ± 1.6 | 0.03 ± 0.01 | 0.02 ± 0.01 |

*Enzyme-BDDFHA complex.

**RESULTS**

*Inactivation of AdoHcy Hydrolase by BDDFHA—*When AdoHcy hydrolase (4 µM) was incubated with a large molar excess of BDDFHA (100 µM), time-dependent loss of activity was observed as shown in Fig. 1. However, the enzyme was not completely inactivated, and the maximal inactivation was 83% of the original enzyme activity. The enzyme was substantially protected from inactivation by BDDFHA by inclusion of the substrate Ado at a concentration equal to the concentration of BDDFHA (100 µM) (Fig. 1). Kinetic analysis using the Kitz and Wilson method gave a Kᵦ value of 3.9 µM, and kᵦmax value of 0.04 min⁻¹ for BDDFHA inactivation of human AdoHcy hydrolase. The enzyme inactivation by BDDFHA was irreversible, i.e., the activity of the inactivated enzyme could not be recovered by dialysis or gel filtration.

**Stoichiometry of Binding and Covalent Modification—**After

20% solvent II with a linear gradient to 60% solvent II over 60 min. Detection of peptides and measurement of radioactivity were the same as described above except that fractions were collected manually peak by peak.

The isolated peptides were sequenced by automated Edman degradation on an Applied Biosystems 473A protein sequencer in the Biochemistry Laboratory at Kansas State University, Manhattan, KS. At each sequencing cycle, the washings from the conversion flask and eluate from the HPLC column were collected and combined for determination of radioactivity.

**RESULTS**

*Inactivation of AdoHcy Hydrolase by BDDFHA—*When AdoHcy hydrolase (4 µM) was incubated with a large molar excess of BDDFHA (100 µM), time-dependent loss of activity was observed as shown in Fig. 1. However, the enzyme was not completely inactivated, and the maximal inactivation was 83% of the original enzyme activity. The enzyme was substantially protected from inactivation by BDDFHA by inclusion of the substrate Ado at a concentration equal to the concentration of BDDFHA (100 µM) (Fig. 1). Kinetic analysis using the Kitz and Wilson method gave a Kᵦ value of 3.9 µM, and kᵦmax value of 0.04 min⁻¹ for BDDFHA inactivation of human AdoHcy hydrolase. The enzyme inactivation by BDDFHA was irreversible, i.e., the activity of the inactivated enzyme could not be recovered by dialysis or gel filtration.
mol of Ade/mol of the inactivated enzyme subunit.

Effect of BDDFHA on E•NAD$^+$.—Inactivation of AdoHcy hydrolase by BDDFHA did not involve the reduction of E•NAD$^+$ to E•NADH as determined by a fluorescence method (data not shown) and by a UV spectroscopic method as shown in Fig. 4. Incubation of AdoHcy hydrolase with BDDFHA resulted in about 60% inactivation of the enzyme in 20 min as shown in Fig. 1, whereas no NADH formation was observed when the reaction was monitored at 340 nm for NADH as shown in Fig. 4, line a. By contrast, incubation of the enzyme with ZDDFA, a type I mechanism-based inhibitor, resulted in complete inactivation of the enzyme and conversion of E•NAD$^+$ to E•NADH in 3 min (Fig. 4, line c). When the enzyme was first incubated with BDDFHA, and then the enzyme-BDDFHA complex (with 30% remaining activity) was incubated with ZDDFA, the enzyme was completely inactivated and about half of the E•NAD$^+$ was converted to E•NADH as shown in Fig. 4, line b.

Isolation and Characterization of [8-3H]BDDFHA-labeled Peptide Fragments.—After incubation of AdoHcy hydrolase with [8-3H]BDDFHA for 2 h at 37 °C, the excess [8-3H]BDDFHA was removed by gel filtration. The enzyme-[8-3H]BDDFHA complex was denatured by 8 M urea and gel-filtered again. The radiolabeled protein was then subjected to tryptic digestion, and the peptides were separated by reverse-phase HPLC. Fig. 5 shows the HPLC chromatogram of the tryptic digested enzyme modified by [8-3H]BDDFHA. The radioactivity was distributed among three major fractions (a, b, and c) as indicated by the bars below the chromatogram. Fraction a (about 25% of total radioactivity) was from incompletely digested large protein fragments, and further digestion of this fraction by trypsin resulted in the formation of fractions b and c (data not shown). Fractions b and c (about 15 and 55% of the total radioactivity, respectively) were collected separately and rechromatographed twice on the same column with different elution conditions. As shown in Fig. 5, inset, fraction c was purified to one major and several minor components with radioactivity associated only with the major component (fraction c'). However, rechromatography of fraction b was not successful enough to obtain completely separated radioactive component required for protein sequencing. The identity of this labeled peptide(s) in fraction b remains unknown. The HPLC-purified radiolabeled peptide (approximately 200 pmol) from fraction c' was subjected to N-terminal protein sequencing. Results from

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**Fig. 2.** Ion-exchange chromatogram of Br$^-$ and F$^-$ ions released from BDDFHA upon incubation with AdoHcy hydrolase. A reaction mixture (3.5 ml) consisting of 28 µM enzyme and 300 µM BDDFHA in buffer A was incubated at 37 °C for various times. At each time point as indicated, an aliquot of the reaction mixture (500 µl) was withdrawn and added to a test tube containing 1.5 ml of 97% ethanol as described under "Experimental Procedures." The released Br$^-$ and F$^-$ ions were quantitated by an ion-exchange column in an HPLC system equipped with a conductivity detector. The chromatogram shows the separation of F$^-$ and Br$^-$ ions from the reaction mixture after a 30 min incubation. Inset, plot of Br$^-$ and F$^-$ ions released versus incubation time. The curves are the fits of the data to an equation for a first-order process.

**Fig. 3.** HPLC chromatogram of the reaction products from incubation of AdoHcy hydrolase and BDDFHA. AdoHcy hydrolase (900 µg) was incubated with BDDFHA (300 µM) in 500 µl of buffer A at 37 °C for various times. At each time point, the reaction was stopped by the addition of 10 µl of 5 M HClO$_4$, and the supernatant from centrifugation was analyzed by HPLC as described under "Experimental Procedures." Ade was eluted at a retention time of 9.685 min. A, HPLC chromatogram of Ade released from BDDFHA; B, HPLC chromatogram of enzyme alone as a control. Inset, plot of Ade released versus incubation time. The curve is the fit of the data to an exponential function.
the first 13 cycles of the protein sequencing revealed that fraction c9 had an amino acid sequence of 1Ser-Lys-Phe-Asp-Asn-Leu-Tyr-Gly-\(X\)-X-Glu-Ser-\(X\)-X-Glu-Ser-Leu, in which \(X\) represents unidentified amino acid residues. The tritium radioactivity was found to be only associated with the unidentified residue \(X\) in cycle number 10. By comparing the amino acid sequencing deduced from the cDNA sequencing encoding human placental AdoHcy hydrolase with the sequence obtained from fraction c9, it was found that fraction c9 contained a fragment of the hydrolase protein from Ser187 to Leu199 with a sequence of Ser-Lys-Phe-Asp-Asn-Leu-Tyr-Gly-Cys-Arg-Glu-Ser-Leu. The amino acid residue associated with radioactivity was then identified as Arg196.

**DISCUSSION**

Pharmacological modulation of intracellular methylation can be achieved through feedback inhibition of methyltransferase activity by AdoHcy (2). Intracellular AdoHcy concentrations can be elevated by decreasing AdoHcy hydrolase activity (27).
Numerous nucleoside analogs capable of reversibly or irreversibly inhibiting AdoHcy hydrolase have been isolated or synthesized as potential antiviral, antiparasitic, antiarthritic, immunosuppressive, and antitumor agents (3–10). More recently, AdoHcy hydrolase inhibitors have been reported to be specially effective against filovirus such as Ebola virus (28).

Many of these nucleoside analogs are type I mechanism-based inhibitors of AdoHcy hydrolase, which inactivate the enzyme by reducing the E-NAD$^+$ to E-NADH in an irreversible manner but not involving covalent linkage between the enzyme and inhibitor. Various attempts have been made to prepare type II mechanism-based inhibitors of this enzyme (14, 15, 19, 21), which would be activated by the enzyme’s catalytic activity and inactivate the enzyme through covalent modification. This type of mechanism-based inhibitor, starting with relatively unreactive structures that are activated only by specific targeted enzymes, often have better pharmacological specificity (29). ZDDFA was designed and synthesized as a potential type II mechanism-based inhibitor (15). Through mechanistic studies on the enzyme inhibition by ZDDFA (20) and its homolog EDDFHA (30), it has been demonstrated that ZDDFA is not a type II mechanism-based inhibitor, instead, it is a pro-inhibitor, which inactivates the enzyme by a type I mechanism. More importantly, with these molecular tools (ZDDFA and EDDFHA), we have demonstrated that AdoHcy hydrolase possesses 5’- and 6’-hydrolytic activities that are independent of the 3’-oxidative activity (20, 30). This finding has led us to the design of BDDFHA which targets the enzyme’s 6’-hydrolytic activity to generate a strong electrophilic center (i.e. acyl fluoride analog) in the active site, which could then react with a juxtaposed protein nucleophile to form a covalent adduct with the enzyme. Evidence obtained in this study strongly supports the conclusion that BDDFHA is the first type II mechanism-based inhibitor of AdoHcy hydrolase, which relies only on the enzyme’s hydrolytic activity for activation.

Scheme 2 contains the proposed mechanism by which BDDFHA inactivates the enzyme. In this mechanism, addition of enzyme-mediated water at the C-6’ position of BDDFHA followed by elimination of Br$^-$ ion results in the formation of homoadenosine 6’-carboxyl fluoride (HACF). HACF then partitions in two ways: (a) attack by a protein nucleophile (e.g. Arg$^{196}$) forms an amide bond after expulsion of F$^-$ ion or (b) depurination releases Ade and forms hexose-derived 6-carboxyl fluoride (HDCF). HDCF is further hydrolyzed to hexose-derived 6-carboxylic acid (HDCA) and F$^-$ ion. In contrast to the type I mechanism-based inhibitors, inactivation of AdoHcy hydrolase by BDDFHA does not involve reduction of E-NAD$^+$ to E-NADH. No NADH was formed during the enzyme inactivation process, and the inactivated enzyme retains its original NAD$^+$/NADH content.

Evidence consistent with the pathway leading to enzyme inactivation includes the observations that incubation of AdoHcy hydrolase with BDDFHA results in release of both Br$^-$ and F$^-$ ions as well as covalent labeling of the enzyme. The first-order rate of Br$^-$ ion release ($k_2 = 0.27 \text{ min}^{-1}$) is greater than the rate of F$^-$ ion release ($k_{app} = 0.098 \text{ min}^{-1}$), in agreement with the prediction that Br$^-$ is a better leaving group than F$^-$. Release of Br$^-$ affords HACF, which contains a strong electrophilic center. Nucleophilic attack at C-6’ of HACF by Arg$^{196}$ results in elimination of F$^-$ ion and covalent linkage between Arg$^{196}$ and C-6’ carboxylic group of HACF, probably via formation of an amide bond. The stoichiometry of covalent labeling of AdoHcy hydrolase by BDDFHA is found to be only 2 mol of inhibitor/mol of the tetrameric enzyme, indicating that two of the four subunits are covalently labeled and the other two are not. The two unlabelled enzyme subunits are still enzymatically viable but with reduced activity. The $k_{cat}$ value for the BDDFHA-inactivated enzyme is 1.2 s$^{-1}$, which is only one-third of that of the native enzyme ($k_{cat} = 3.6 \text{ s}^{-1}$, in the hydrolytic direction). From these $k_{cat}$ values, the calculated remaining enzyme activity theoretically should be 16.7%, which agrees well with the experimental result (approximately 20%). This remaining enzyme activity can be further inhibited by ZDDFA (Table I) via conversion of the E-NAD$^+$ to NADH (Fig. 2, line b). Covalent modification of Arg$^{196}$ on two of the subunits by BDDFHA appears to cause a conformational change that disrupts the 3’-oxidative activity of the two unmodified subunits. This conformational change is transmitted, presumably through intersubunit contact, to the neighboring subunit which loses its catalytic activity toward BDDFHA but retains reduced enzymatic activity toward the substrate AdoHcy or the inhibitor ZDDFA. Similar inactivation of two subunits that affected the other two subunits in a tetrameric enzyme was also observed by Abeles et al. (31) upon inactivation of calf liver AdoHcy hydrolase with 2’-deoxyadenosine. Parry et al. (21) also reported that inactivation of AdoHcy hydrolase by an acetylenic analogue of adenosine resulted in covalent modification of two subunits. This acetylenic analog of adenosine, which also appears to be a type II inhibitor, needs to be activated by the enzyme’s 3’-oxidative activity leading to conversion of two equivalents of NAD$^+$ to NADH. Parry et al. (21) reported that the two remaining equivalents of NAD$^+$ in the tetramer were “released from the enzyme.” These results suggest subunit-subunit interaction involving the binding of the cofactor NAD$^+$. Evidence in support of the nonlethal pathway observed with BDDFHA derives from the observation that incubation of AdoHcy hydrolase with BDDFHA results in the formation of Ade (0.6 mol/enzyme subunit) (Fig. 3). Moreover, the difference in the stoichiometries of total F$^-$ ion release (0.9 mol/enzyme subunit) and covalent modification (0.5 mol/enzyme subunit) suggests that there must be other pathways that produce F$^-$ ion. The proton on C-5’ of HACF could be abstracted by a base from the enzyme, which could result in depurination via a retro-Michael addition process to generate Ade and HDCF.
Water attack at C-6 of HDCF results in the release of F⁻ ion and formation of HDCA. Combination of these pathways produces about equal mol of Br⁻ and F⁻ ions, which is approximately equal to the sum of the Ade and covalent incorporation. Based on equation (2), \( k_d \) is calculated to be 0.057 min⁻¹, which is equal to \( k_b (0.056 \text{ min}^{-1}) \). Therefore, the partition ratio of the nonlethal to lethal pathways, or \( k_b/k_d \), is approximately 1:1, indicating one lethal event (enzyme inactivation via covalent modification) for every two enzymatic turnovers.

An important criteria for a mechanism-based inhibitor is the formation of a covalent linkage between the enzyme and the inhibitor. Arg⁹¹⁹ was identified as the major nucleophile that attacks the electrophilic center in HACF resulting in formation of a covalent linkage. This most likely is an amide bond between C-6' of HACF and the guanidinium group of Arg⁹¹⁹. It is possible that Arg⁹¹⁹ is located at the enzyme active site close to Cys⁹¹⁹, which was identified as an essential residue at the active site of the enzyme. It may play a role in maintaining the 3'-reduction potential for regeneration of the NAD⁺ form of the enzyme from the NADH form (24). Arg⁹¹⁹ is also next to Glu⁹¹⁹, which was demonstrated to be at the enzyme active site by limited proteolytic studies (32). In addition, Arg⁹¹⁹ is only about 10 amino acid residues from a peptide (Val¹⁷⁵–Lys¹⁸⁶) located in the Ade-ring-binding region (33) and 14 amino acid residues from the NAD⁺ binding region (34). However, it is also possible that Arg⁹¹⁹ is not positioned closely to the C-5' or C-6' of HACF bound in the enzyme active site, but is located in such a position that allows it to react with HACF dissociating from the active site. This may explain the observation that multiple radiolabeled tryptic peptides (fragments b and c in Fig. 5) were found after incubation of the enzyme with [8-³H]BDDFHA, indicating that Arg⁹¹⁹ is not the only amino acid residue reacting with C-6' of HACF. In addition, results from this study do not tell if Arg⁹¹⁹ is the base that abstracts the C-4' proton to form a Michael acceptor in the normal enzymatic catalysis of AdoHcy hydrolyse in the Palmer-Abeles mechanism (11).

To our knowledge, BDDFHA is the first type II mechanism-based inhibitor of AdoHcy hydrolase that relies only on the enzyme's hydrolytic activity for activation. This high specificity of enzyme inactivation should diminish cytotoxicity resulting from other metabolic effects induced by most type I mechanism-based inhibitors (23). The antiviral activity and cytotoxicity of this first type II mechanism-based inhibitor are currently under investigation.²

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