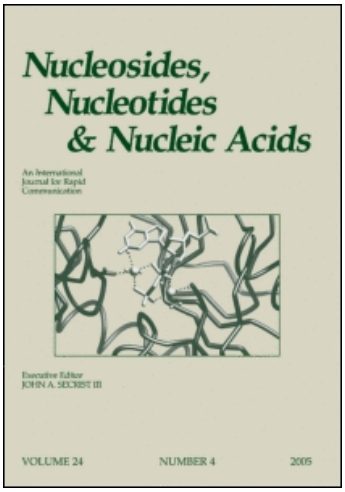

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RATIONAL APPROACHES TO THE DESIGN OF MECHANISM-BASED INHIBITORS OF *S*-ADENOSYLHOMOCYSTEINE HYDROLASE

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Abstract: Crucial to the rational design of inhibitors of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase was the elucidation of its mechanism of catalysis by Palmer and Abeles (*J. Biol. Chem.* **254**, 1217-1226, 1979). This mechanism involves an NAD^+-dependent oxidation (oxidative activity) of the 3'-hydroxyl group of AdoHcy followed by elimination of homocysteine (Hcy) to form 4',5'-didehydro-3'-keto-Ado. Addition of water at the 5'-position (hydrolytic activity) of this tightly bound intermediate followed by an NADH-dependent reduction results in the formation of adenosine (Ado). Many inhibitors of this enzyme have been shown to serve as substrates [*e.g.*, 9-(*trans*-2-*trans*-3-dihydroxycyclopent-4-en-1-yl)adenine, DHCeA)] for the oxidative activity of AdoHcy hydrolase, affording the 3'-keto-derivative (*e.g.*, 3'-keto-DHCeA), which is tightly bound to the enzyme, and converting the enzyme from its active form (NAD^+) to its inactive form (NADH) (Type I mechanism-based inhibitors; Wolfe and Borchardt, *J. Med. Chem.* **34**, 1521-1530, 1991). More recently, substrates [*e.g.*, (*E*)-5',6'-didehydro-6'-deoxy-6'-fluorohomoadenosine, EDDFHA] for the hydrolytic activity of AdoHcy hydrolase have been identified by our laboratories. Identification of hydrolytic substrates affords a new strategy for the design of more potent and more specific inhibitors of AdoHcy hydrolase.

In recent years, AdoHcy hydrolase (EC 3.3.1.1) has become an attractive target for drug design because inhibitors of this enzyme have been shown to exhibit antiviral,[1-4]

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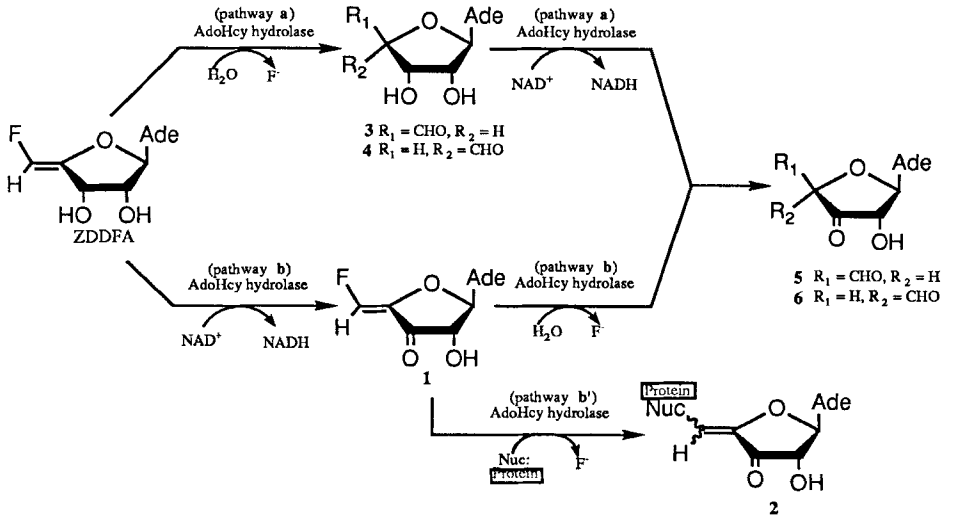
antiparasitic,[5,6] antiarthritic,[7] and immunosuppresive[8,9] effects. These therapeutic effects can in most cases be related to the ability of AdoHcy hydrolase inhibitors to cause an intracellular accumulation of AdoHcy resulting in inhibition of crucial AdoMet-dependent methylation reactions. These relationships are particularly well established for the antiviral effects of AdoHcy hydrolase inhibitors. In fact, a linear correlation between log IC_{50} values (the concentration which inhibits viral replication by 50%) for a series of AdoHcy hydrolase inhibitors and their log K_i values for inhibition of AdoHcy hydrolase has been observed.[1,10]

Significant progress has been made in the rational design of inhibitors of AdoHcy hydrolase since the elucidation of the mechanism of the enzyme catalysis by Palmer and Abeles.[11] This mechanism involves an NAD^+-dependent oxidation (oxidative activity) of the 3'-hydroxyl group of AdoHcy followed by elimination of Hcy to form 4',5'-dehydro-3'-keto-Ado. Addition of water at the 5'-position (hydrolytic activity) of this tightly bound intermediate followed by an NADH-dependent reduction results in the formation of Ado. Many of the potent inhibitors of this enzyme have been designed to serve as substrates for the C3' oxidative activity of AdoHcy hydrolase, affording the 3'-keto derivative of the inhibitor, which is tightly bound to the enzyme, and converting the enzyme from its active form (NAD^+) to its inactive form (NADH). Inhibition of AdoHcy hydrolase by this type of inhibitor is generally irreversible since the 3'-keto intermediates are not substrates for the C5' hydrolytic activity of the enzyme. These catalytic interactions between the enzyme and the inhibitor terminate at the half-cycle of the overall enzyme reaction (oxidative only), which irreversibly keeps the enzyme in its NADH form. This in turn tightly traps the inhibitor inside the active site of the enzyme because of the conformational change associated with conversion from the NAD^+ form to the NADH form.[12] Inhibitors, which inactivate AdoHcy hydrolase by reducing the enzyme-bound NAD^+ ($E{\cdot}NAD^+$) to E·NADH in an irreversible manner but do not covalently bind to the enzyme, are referred as Type I mechanism-based inhibitors[3]. Good examples of this type of inhibitor are 9-(*trans*-2-*trans*-3-dihydroxycyclopent-4-en-1-yl)adenine (DHCeA) and 9-(*trans*-2-*trans*-3-dihydroxycyclopentanyl)adenine (DHCaA), which have K_i and k_{inact} values of 85 nM and 0.15 min^{-1} and 33 nM and 0.03 min^{-1}, respectively, against recombinant human placental AdoHcy hydrolase.[13]

Efforts have also been made in the design and synthesis of potential Type II mechanism-based inhibitors of AdoHcy hydrolase. Type II mechanism-based inhibitors of this enzyme are envisoned as compounds that are catalytically activated and subsequently become covalently bound to the enzyme.[3] Initial attempts to prepare Type II mechanism-based inhibitors tried to exploit the oxidative activity of the enzyme to generate an electrophilic site on the inhibitor that could react with a protein nucleophile.[3]

McCarthy and co-workers,[14-16] based on the observation by Palmer and Abeles[11] that 4',5'-didehydro-5'-deoxyAdo is a substrate for AdoHcy hydrolase, synthesized vinyl fluoride analogs of this nucleoside, *e.g.*, (*Z*)-4',5'-didehydro-5'-deoxy-5'-fluoroadenosine (ZDDFA), as potential mechanism-based inhibitors. ZDDFA was shown to be a potent inhibitor of AdoHcy hydrolase.[14-16] In addition to being a potent inhibitor, ZDDFA was of interest mechanistically because it not only reduces the E·NAD$^+$ to E·NADH, but also releases fluoride ion quantitatively.[15,17] These results suggested that ZDDFA might be the first example of a Type II mechanism-based inhibitor of AdoHcy hydrolase that functions by generating a chemically reactive intermediate (3'-keto-ZDDFA, **1**, Scheme 1) at the enzyme active site, which can then react with a protein nucleophile (pathway b', Scheme 1) to form a covalent adduct with the protein (**2**, Scheme 1).[3,14,17] Alternatively, it was suggested that 3'-keto-ZDDFA (**1**) could react with enzyme-sequestered water, releasing fluoride ion and generating the 3'-keto-5'-carboxaldehydes **5** and **6** (pathway b, Scheme 1). It was recently shown by our laboratories that the mechanism of inactivation of AdoHcy hydrolase by ZDDFA involves rapid addition of water at the 5'-position of ZDDFA (hydrolytic activity) and elimination of fluoride ion, resulting in the formation of the 5'-carboxaldehydes **3** and **4** (pathway a, Scheme 1).[17] The 5'-carboxaldehydes are oxidized (oxidative activity) in a slower step to the 3'-keto-5'-carboxaldehydes **5** and **6** (Scheme 1) by reduction of E·NAD$^+$ to E·NADH. Intermediate carboxaldehydes **3** and **4** were synthesized independently and proven to be potent Type I mechanism-based inhibitors.[18] Carboxaldehyde **3** and ZDDFA have identical K_i values of 40 nM, but the k_{inact} value of 0.67 min^{-1} for carboxaldehyde **3** is 8 times greater than that of ZDDFA.[17] These results show that ZDDFA is simply a "pro-inhibitor" for a Type I mechanism-based inhibitor (Ado-5'-carboxaldehyde, **3**) of AdoHcy hydrolase. The unique aspect of this mechanism is that the conversion of the "pro-inhibitor" (ZDDFA) to the "inhibitor" (Ado-5'-carboxaldehyde, **3**) actually occurs at the enzyme active site, utilizing the hydrolytic activity of the enzyme. These observations were particularly significant because they showed for the first time that the "hydrolytic activity" of the enzyme can function independently of the "oxidative activity."

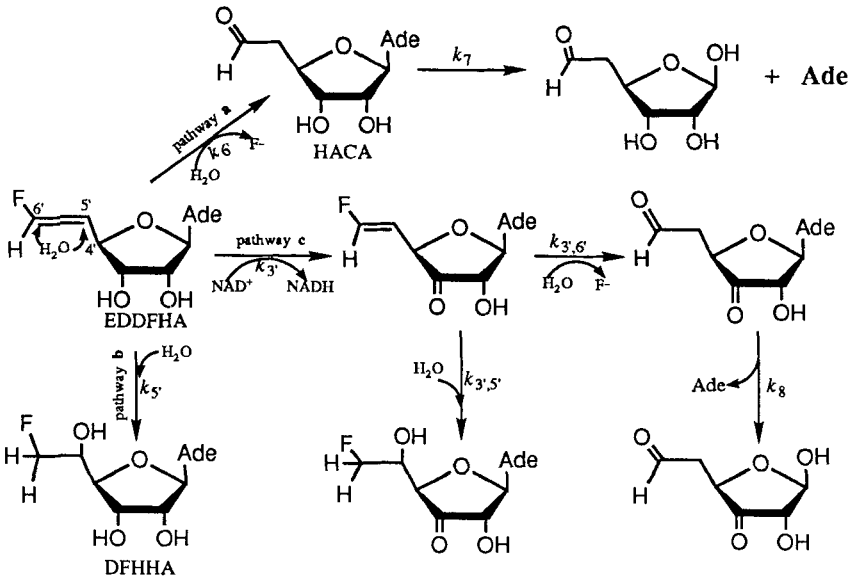
It is interesting to note that the carbocyclic analog of ZDDFA [*e.g.*, (*Z*)-4',5'-didehydro-5'-deoxy-5'-fluoroaristeromycin, ZDDFAri] was shown not to be a substrate for the hydrolytic activity of the enzyme since incubation of ZDDFAri with AdoHcy hydrolase did not result in the release of fluoride ion.[19] It is possible that enzyme-mediated protonation of the ribosyl ring oxygen of ZDDFA could enhance the electrophilicity of the 5'-carbon, thus the 5'-position would be more susceptible to attack by water sequestered at the active site of the enzyme. Nevertheless, both ZDDFAri and the carbocyclic analog (*e.g.*, Ari-5'-carboxaldehyde) of Ado-5'-carboxaldehyde (**3**) were



Scheme 1. Possible mechanism by which ZDDFA inactivates AdoHcy hydrolase

shown to be potent Type I mechanism-based inhibitors of AdoHcy hydrolase with K_i and k_{inact} values of 87 nM and 0.05 min^{-1} for ADDFAri and 273 nM and 1.2 min^{-1} for Ari-5'-carboxaldehyde,[19, 20] respectively, when assayed against the rat liver enzyme.

More recently, our laboratories have shown that (*E*)-5',6'-didehydro-6'-deoxy-6'-halohomoadenosines (EDDHHAs) (see structures in FIG. 1) are also substrates for the hydrolytic activity of the enzyme and posses anticancer and antiviral activities that parallel the enzyme inhibition.[21-23] The hydrolytic activity for EDDHHAs is defined as the ability of AdoHcy hydrolase to catalyze addition of water at the 5',6'-position of EDDHHAs. Scheme 2 shows the mechanism by which the fluoride derivative (EDDFHA) is processed by AdoHcy hydrolase. Incubation of EDDFHA with AdoHcy hydrolase produces a large molar excess of hydrolytic products [*e.g.*, fluoride ion, Ade derived from chemical degradation of homoadenosine 6'-carboxaldehyde (HACA), and 6'-deoxy-6'-fluoro-5'-hydroxyhomoadenosine (DFHHA)] accompanied by a slow irreversible inactivation of the enzyme.[22] The enzyme inactivation was shown to be time-dependent, biphasic and concomitant with the reduction of the E·NAD^+ to E·NADH. The reaction of EDDFHA with AdoHcy hydrolase was shown to proceed by three pathways: pathway a, water attack at the 6'-position of EDDFHA and elimination of fluoride ion results in the formation of HACA, which degrades chemically to form Ade; pathway b, water attack at the 5'-position of EDDFHA results in the formation of DFHHA; and pathway c, oxidation of EDDFHA results in the formation of the NADH form of the



Scheme 2. Mechanism of inactivation of AdoHcy hydrolase by EDDFHA

enzyme (inactive) and 3'-keto-EDDFHA, which could possibly react with water at either the C5' or C6' positions. The partition ratios among the three pathways were determined to be $k_{3'}$:$k_{6'}$:$k_{5'}$ = 1:29:79 with one lethal event (enzyme inactivation) occurring every 108 nonlethal turnovers.[22] The ratios of $k_{5'}/k_{6'}$ strongly depend on the properties of the halogen at the C6' position and were shown to be in the order of F>Cl>Br>I (Table 1). Similarly, the partition ratios [the ratios of turnover events to inactivation events, *i.e.*, $(k_{5'}+k_{6'})/k_{3'}$] were also in the order of F>Cl>Br>I (Table 1). The large partition ratio (108) for

EDDFHA makes it a "fairly specific" substrate for measuring the hydrolytic activity of the enzyme. The significantly greater electronegativity of fluorine relative to that of the other halogens has been invoked to rationalize the enhancement of the acidity of hydrogens on carbon atoms β to fluorine. In contrast, the other halogens enhance the acidity of geminal hydrogens on the α carbons[23].

Our laboratories have recently shown that in addition to ZDDFA and EDDHHAs serving as substrates for the hydrolytic activity of AdoHcy hydrolase, 4',5'-didehydro-5'-methoxyAdo (DMOA, FIG. 1)[24] and Ado-5'-carboxaldehyde oxime (ACAO, FIG. 1)[25] are also potent inhibitors of AdoHcy hydrolase through a mechanism involving the hydrolytic activity. Inactivation of the enzyme by DMOA and ACAO was shown to

Table 1: Kinetic constants and partition ratios of EDDHHAs toward recombinant human placental AdoHcy hydrolase[21-23]

Inhibitors	Inhibition constants			Partition ratios	
	K_i (nM)	k_{inact} (min^{-1})	k_{inact}/K_i (M^{-1} min^{-1})	$k_{5'}/k_{6'}$	$(k_{5'} + k_{6'})/k_{3'}$
EDDIHA	96 ± 7.6	0.058 ± 0.007	6.04×10^5	0.08	7.0
EDDBrHA	134 ± 6.4	0.037 ± 0.004	2.76×10^5	0.11	12.8
EDDClHA	110 ± 8.2	0.015 ± 0.004	1.36×10^5	0.37	38.9
EDDFHA	1300 ± 56	0.011 ± 0.006	8.4×10^3	2.75	108

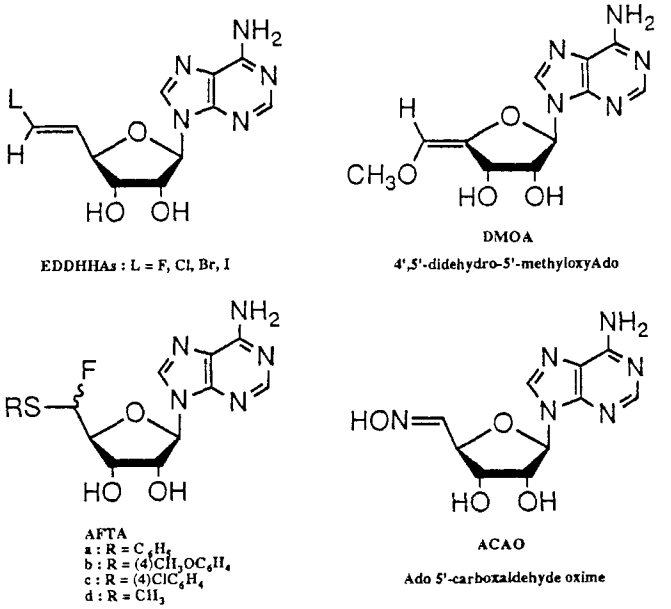
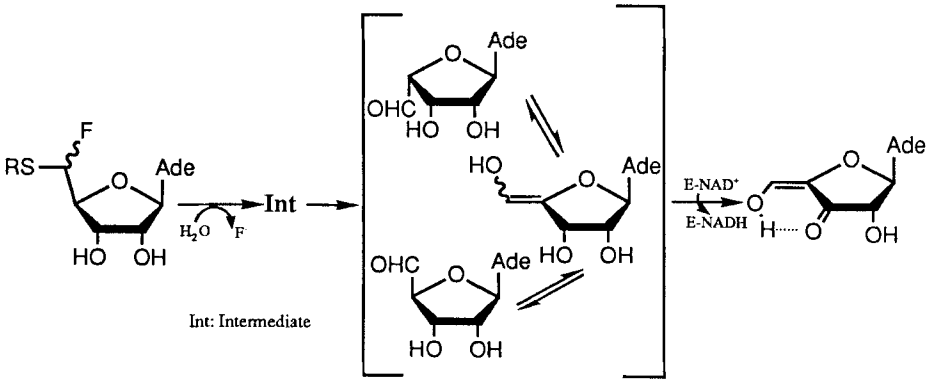


FIG. 1. AdoHcy hydrolase inhibitors



Scheme 3. Mechanism of inactivation of AdoHcy hydrolase by AFTA

follow the ZDDFA-type mechanism for inactivation of AdoHcy hydrolase, *i.e.*, DMOA and ACAO were first converted to the active species by the hydrolytic activity of the enzyme. The resulting Ado-5'-carboxaldehyde then functions as a Type I mechansim-based inhibitor of the enzyme. The K_i and k_{inact} values for DMOA and ACAO toward recombinant human AdoHcy hydrolase were determined to be 1.59 μM and 0.13 min^{-1} and 0.22 μM and 0.13 min^{-1}, respectively.

Recently, we have shown that the potent Type I mechanism-based inhibitor Ado-5'-carboxaldehyde can also be generated non-enzymatically from 5'-S-(alkyl and aryl)-5'-fluoro-5'-thioadenosines (AFTA, FIG. 1). The AFTAs were unstable in aqueous solutions and spontaneously degrade to Ado-5'-carboxaldehyde via a consecutive reaction mechanism of AFTA $\xrightarrow{k_1}$ Int $\xrightarrow{k_2}$ Ado-5'-carboxaldehyde[24] as shown in Scheme 3. The k_1 and k_2 values for AFTA (b) were estimated to be 0.1 min^{-1} and 0.15 min^{-1}, respectively. Rates for solvolysis of the AFTAs were in the order AFTA (d) >>AFTA (b) ≥AFTA (a) > AFTA (c). All of the AFTAs were shown to produce time- and concentration-dependent inactivation of AdoHcy hydrolase. Unlike ZDDFA, which functions as a prodrug of the active 5'-aldehyde-derived Type I mechanism-based inhibitors, the AFTAs (which are two-stage synthetic precursors, oxidation and thermolysis of the fluorovinyl analogs) appear to function as "spontaneuous solvolysis prodrugs" of the same inhibitor(s).

Based on the mechanism elucidated for the ZDDFA, EDDHHAs, DMOA and ACAO-induced inactivation of AdoHcy hydrolase, we feel that the hydrolytic activity of the enzyme can be used to our advantage in designing inhibitors of this enzyme by two strategies: a prodrug strategy which uses the hydrolytic activity of the enzyme to catalyze

formation of Type I mechanism-based inhibitors (*e.g.*, ZDDFA conversion to Ado-5'-carboxaldehyde); and a k_{cat} or sucide inhibition strategy which uses the hydrolytic activity of the enzyme to catalyze formation of strong electrophiles that could covalently modify the enzyme (Type II mechanism-based inhibition).

Conclusions: Mechanistic studies of inactivation of AdoHcy hydrolase by ZDDFA and EDDHHAs have shown that AdoHcy hydrolase possesses two catalytic activities, *i.e.*, hydrolytic activity and oxidative activity, which function independently of each other. This hydrolytic activity of the enzyme might be used to our advantage in designing Type II mechanism-based inhibitors of AdoHcy hydrolase by using it to trigger the formation of a strong electrophilic site on the molecule, which could then react with a protein nucleophile to form a covalently bound inhibitor-enzyme adduct.

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