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Rational Design of a Profluorescent Substrate for S-adenosylhomocysteine Hydrolase and its Applications in Bioimaging and Inhibitor Screening

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KEYWORDS: S-adenosylhomocysteine hydrolase; Fluorogenic substrate; Kinetic study; Bioimaging; Inhibitor screening.

ABSTRACT: S-adenosylhomocysteine hydrolase (SAHase) is a cellular enzyme that plays a key role in the methylation process, and a potential drug target in the discovery of antiviral and anticancer agents. There is increasing interest in determining its activity in the biological and clinical fields with chemosensors but with limited success so far. Herein, we designed and developed for the first time an off/on-type of fluorogenic substrate (NADE) that is directly responsive to SAHase activity. NADE used 1,8-naphthalimide as the signal reporter and adenosine (Ade) as the reaction center; removal of the Ade moiety enhanced the fluorescence by>10-fold. Kinetic study showed that NADE followed a non-Michaelis-Menten pattern that corresponded to the allosteric behavior of SAHase. NADE showed excellent selectivity and functioned efficiently in cells, allowing the microscopic imaging of SAHase activity. NADE can also be used to identify and measure the effectiveness of inhibitors in a markedly superior way. In a word, NADE would be broadly useful in clinical applications and academic studies.

Introduction

In eukaryotes, S-adenosylhomocysteine hydrolase (SAHase) serves as the sole vehicle for the removal of S-adenosylhomocysteine (AdoHcy)[1]. AdoHcy is the coproduct of all biological transmethylation reactions requiring adenosylmethionine (AdoMet) and a potent feedback inhibitor of some AdoMet-dependent methyltransferases[2]. As a result, SAHase regulates all AdoMet-dependent biological transmethylation reactions and thus affects all facets of biometabolism[3,4]. SAHase dysfunction is linked to numerous pathologies, such as fatty liver[5], cancer[6], renal insufficiency[7], diabetic nephropathy[8], as well as neurological and vascular disorders[9,10]. SAHase deficiency[11-13] is recognized as a rare genetic disorder, typically leading to severe pathological consequences and death in childhood. However, identifying the function and regulation mechanism of SAHase in complex pathogeneses remains challenging.

Thus, the development of appropriate SAHase probes is urgently needed. Fluorescent antibody labeling is an efficient tool to track SAHase in living cells[14]. However, this method does not indicate SAHase activity and is time consuming, labor intensive, and expensive. In contrary, reaction-based small-molecule fluorescent probes represent a promising technology owing to its simplicity and convenience[15-17]. In recent years, several chemosensors were employed for SAHase determination[18-23] (Scheme 1). These probes can indirectly indicate SAHase activity by responding to Hcy, the metabolite of AdoHcy. Others probes can indicate SAHase activity by responding to Ade[24]. In practice, a major limitation for such an approach is that a direct association between the probe and SAHase cannot be readily established in in vivo studys because Hcy and SAHase coexist in cells. Therefore, developing a fluorogenic substrate is well-suited for addressing these issues.

Experimental Section

General Information

All commercially available reagents, solvents were purchased from Sigma Aldrich, *J&K* chemicals, Aladdin, Energy Chemical, selleck, cayman and used without further purification. SAHase was obtained from BeijingAmbitionBiotechnology Co, Ltd.(ABXBIO) Spectroscopic data was measured on the Thermo Scientific™ Varioskan™ Flash Multimode Reader (Thermo, USA). Data analysis was performed using GraphPrism 5.0. LC result was performed on a UFLC spectrometry system. NMR spectra were recorded on a Bruker 400 MHz/500 MHz spectrometer. Both were available at DICP (Dalian Institute of Chemical Physics) research facilities center. ^{1}H and ^{13}C chemical shifts are reported relative to the residual solvent peak and are given in ppm. s: singlet, d: duplet, t: triplet, q: quartet, bs: broad signal, m: multiplet.

Molecular Docking

Molecular docking study was performed with autodock 4.2 program[25]. Here, the X-ray crystallographic structure of SAHase (human enzyme complexed with DHCeA, PDB code: 1A7A)[3] was selected for docking analysis. The beta chain of protein and heteroatoms were removed from original PDB file. The ligand (Sahhn) was docked into the active site of SAHase. During the docking process, the Lamarckian genetic algorithm (LGA) was applied to the conformational search for the protein-ligand binding structure. Among a series of docking parameters, the grid size was set to be 60 Å×60 Å×60 Å, and the grid space was the default value of 0.375 Å. The pose with the lowest free energy of binding was selected as the best binding mode. The predicted binding energy for Sahhn was -10.18 kcal·mol^{-1}.

Computational method

For the theoretical study of excited state photophysics of NADE and NASH, density functional theory (DFT) method was carried out and the Becke's three-parameter hybrid exchange functional with Lee-Yang-Parr gradient-corrected correlation (B3LYP functional) was

used as the method of choice.[26-30] Geometries for NADE and NASH were fully optimized without symmetry constraints. All the local minima were confirmed by the absence of an imaginary mode in vibrational analysis calculations. Then, we calculated the vertical excitation energies based on the optimized geometries of the two molecules.

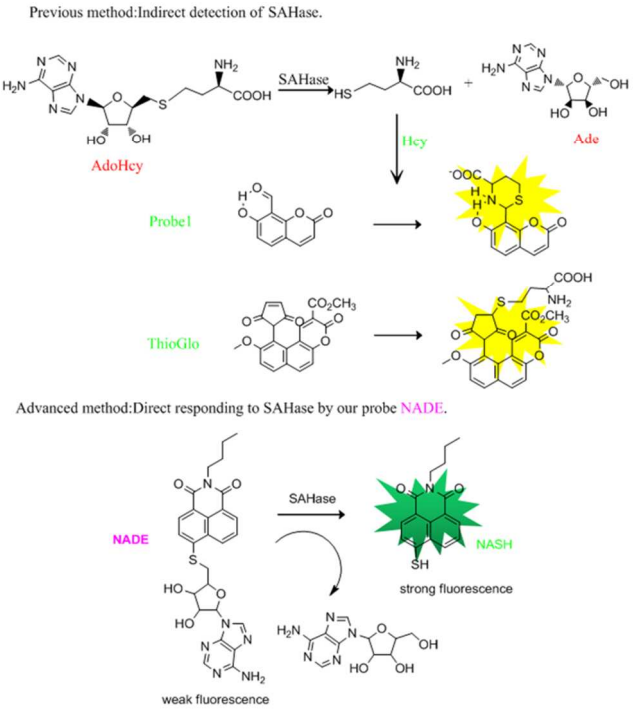
General Procedure for Monitoring SAHase Activity

Stock solutions of SAHase(100mg/mL, 10mg/mL, 1mg/mL) were prepared in distilled water and stored in frozen aliquots at -20℃. Slow thaw on ice just before experiment and each one could only be thawed once. Stock solution of NADE (5mM, 500μM) was prepared in DMSO. Both enzyme and probe are diluted in phosphate buffer solution (pH=7.4) to specified concentration when used. Fluorescence spectra or time-dependent fluorescence intensity at specified wavelength was recorded using Thermo Scientific™ Varioskan ™ Flash Multimode Reader (Thermo, USA).

Cell studies and Bioimaging Study

HL-60 cells lines were obtained from the CAS (Chinese Academy of Sciences) Cell Bank. and cultured in IMDM(Hyclone) medium supplemented with 20% (v/v) fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2 at 37℃. The cells in 35 mm×12 mm glass bottom cell culture dishes were set at a density of 2.5×10^5 /mL. The stock solution of NADE in DMSO (500μM) was diluted with phosphate buffered saline solution (100 mM, pH 7.4, 138 mM NaCl). Unless otherwise mentioned, final concentration of probe NADE applied to cells is 5μM. Confocal fluorescence images (Ex. 405nm, Em. 467-517nm) were observed with Olympus FV1000 confocal laser-scanning microscope with an objective lens (×100).

Results and Disscussion



Scheme 1. Mechanism of previous S-adenosylhomocysteine Hydrolase (SAHase) probes in comparison with proposed mechanism of our probe NADE.

Design Strategy and Sensing Mechanism

Our strategy to develop a SAHase-specific fluorescence probe relied on the design of a pro-fluorescent substrate for SAHase (Scheme 1). In this regard, 1,8-naphthalimide was selected as a signal transducer because of its good photostability, high brightness, and ease of incorporation by an enzyme cavity. An adenosine moiety, which acts as the recognition center of the native substrate (AdoHcy) of SAHase, was introduced to render the artificial substrate recognizable by the enzyme. The dye and the adenosine moiety were joined by a sulfur atom to form a thioether bond, and this functionality served as the reactive center of SAHase-catalyzed hydrolytic

reactions. In addition, the reactive center was designed at the 4-position of the 1,8-naphthalimide skeleton (Scheme 1, NADE) because the fluorescence of this dye is sensitive to substituents at this position[31]. Herein, computational methods (B3lyp/6-311g (d,p)) were used to figure out the excited state of NADE and NASA. Results showed that photo-induced electron transfer (PET) took place from the NI moiety to the Ade moiety in NADE, corresponding to the orbital transition from HOMO to LUMO (99.9%), while not occur in NASH, corresponding to the orbital transition from HOMO to LUMO (94.7%) (Figure 1, detailed information are shown in supporting information). Hence, the artificial substrate NADE was fluorescently silent (quenched by PET); however, once recognized and hydrolyzed by SAHase, the fluorescence will be enhanced significantly and demonstrated SAHase activity in real time.

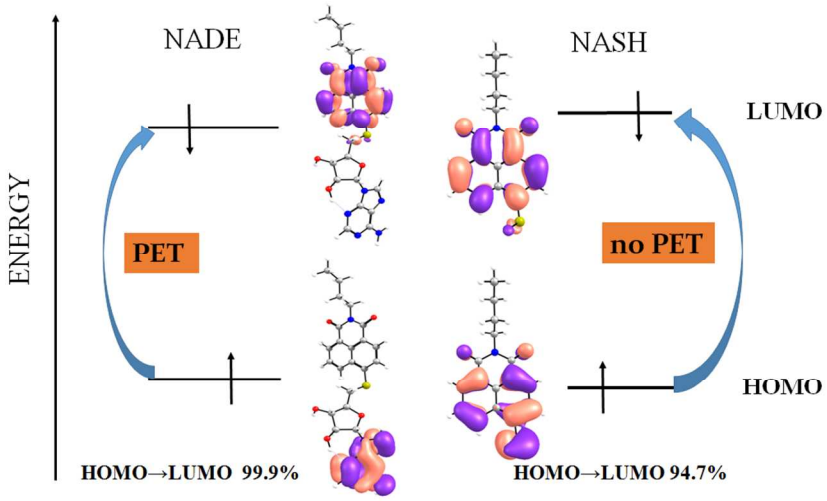


Figure 1. Computational result revealed the mechanism of fluorescence enhancement of NADE upon hydrolyzing.

Docking Simulation

To test the feasibility of our design, we first performed a molecular modeling study to explore the mode of molecular interaction of NADE in the active site. Figure 2 shows that NADE can be well docked into the active cavity of SAHase (human enzyme complexed with DHCeA, PDB code: 1A7A). Several H-bonds obviously formed between NADE and SAHase. The C_2'H in the furan moiety could form an H-bond with Lys-186 and Asp-190, whereas the C_3' H could form an H-bond with Thr-157 and wat-436. N_6' H, N_1, and N_7 in the adenine moiety formed an H-bond with Glu-59, Thr-57, and His-353, respectively. In addition, carbanyl O in the naphthalimide moiety formed an H-bond with Thr-158, Thr-159, and His-301. These H-bonds stabilized NADE in the active site with a proper conformation, similar to that of Adohcy[32, 33] or DHCeA[3]. This conformation allowed for convenient H transfer from the substrate to NAD^+, where enzymatic hydrolysis was initiated. Moreover, our modeling results showed that a H_2O molecule (wat-436) existed in the vicinity of the ribose moiety. According to the enzyme mechanism proposed by Palmer and Abeles[34], a neighboring H_2O molecule is essential to ensure an efficient enzymatic reaction by trapping 4', 5'-didehydro-3-ketoadenosine, an intermediate formed by the β-elimination reaction of the H transfer product. These results suggest that NADE could be well recognized by SAHase and that NADE is likely to be selected as the SAHase substrate.

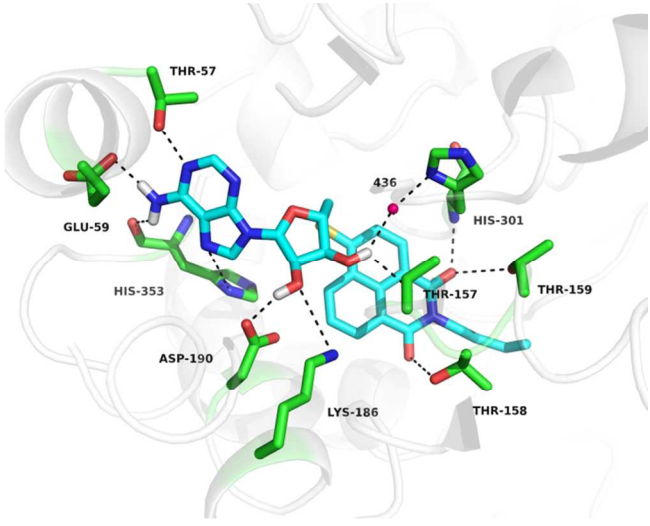


Figure 2. Docking simulations showed the detail of NADE binding site in SAHase active pocket. Hydrogen bonds are represented by dotted black lines, and water molecules are represented by red ball.

Synthesis of NADE and Product Identification

To verify the design strategy and modeling results, we synthesized NADE (Scheme S1, see ESI, ^{1}H NMR, ^{13}C NMR) and tested the enzyme with it. The enzymatic hydrolytic reactions of NADE were investigated with HPLC (Figure S5). NADE was stable in phosphate buffer solution (PBS, pH = 7.4), and the retention time of the main peak was 5 min. However, a new peak with a retention time of 8 min appeared when SAHase was added into the NADE solution. The retention time of this new peak was consistent with that of NASH, the expected product of the hydrolytic reaction. In addition, the appearance of the new peak was blocked when 3-deazaneplanocin A (DZNep), a commonly used inhibitor of SAHase, was added into the assay solutions. The enzymatic reaction was further performed in a preparative scale, and the product was separated and identified as NASH by ^{1}H NMR (SI). These results suggest that NADE served as an excellent substrate for SAHase.

Spectral Properties of NADE toward SAHase

Next, we asked whether the hydrolysis of this artificial substrate could indicate enzymatic activity through changes in its fluorescence. The spectroscopic properties of NADE were examined under simulated physiological conditions (10mM PBS, pH 7, Figure3). NADE showed

a considerably weak fluorescence with an emission maximum at 480nm (Figure S2). However, the fluorescence intensity at 470nm increased dramatically when NADE was subjected to SAHase-catalyzed hydrolytic reactions. The fluorescence enhancements were strongly dependent on SAHase concentrations (Figures 3a and 3b). Figure 3d shows that a good linear relationship (R^2=0.99) was obtained between the fluorescent intensity (470nm emission) and enzyme concentrations of 0-1.0U/mL. These results demonstrate that NADE served as an efficient pro-fluorescent substrate for SAHase and could be used to indicate SAHase activity. The detection limit was 0.019U/mL (Figure S6).

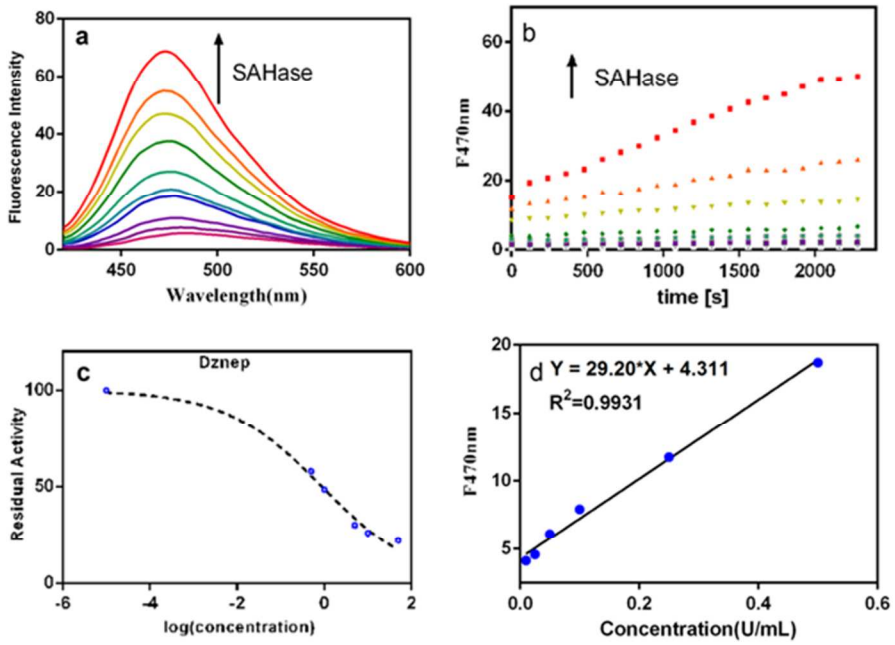


Figure 3. a. Emission spectra of NADE upon addition of 0.2-100U/mL SAHase. b. Time-dependent fluorescence enhancement as a function of 0.2-100U/mL SAHase. c. Plots of the effect of DZNep on NADE hydrolysis in 5U/mL SAHase solution. d. Linear relationship between SAHase and F_{470}nm.

Enzyme Kinetics of NADE

The result of our modeling confirmed that NADE bound with the SAHase active site, a requirement for catalytic activity. In addition, being an enzyme consisting of four identical subunits, SAHase undergoes allosteric dynamics, as revealed by X-ray and NMR experiments, as well as by previous mutational studies [34]. A kinetic study also serves as an efficient tool to reveal the allosteric behavior of multisubunit enzymes[35]. In the present study, we determined whether NADE follows a non-Michaelis-Menten pattern that corresponds to allosterism. We then obtained the kinetic constant (K_m, V_{max}) for the SAHase-catalyzed hydrolysis of the probe NADE by well fitting into a non-Michaelis-Menten model (biphasic model) with a high goodness of fit (R^2=0.9997, equation is shown in SI, Prism 4; GraphPad Software Inc., San Diego, CA; Figures 4 and S7). The apparent K_m values of the hydrolytic reaction were approximately 0.31 and 72.43μM in SAHase, whereas the V_{max} values were 0.02 and 2.02pmol/min/nmol. The apparent non-Michaelis-Menten pattern suggests that NADE serves as a good substrate and further verifies our design strategy. This property renders NADE with incomparable advantages over previous probes, which cannot interact directly with SAHase. NADE enables the identification of the enzyme mechanism of SAHase through kinetic studies, which can provide deep insight into the SAHase-substrate mechanism.

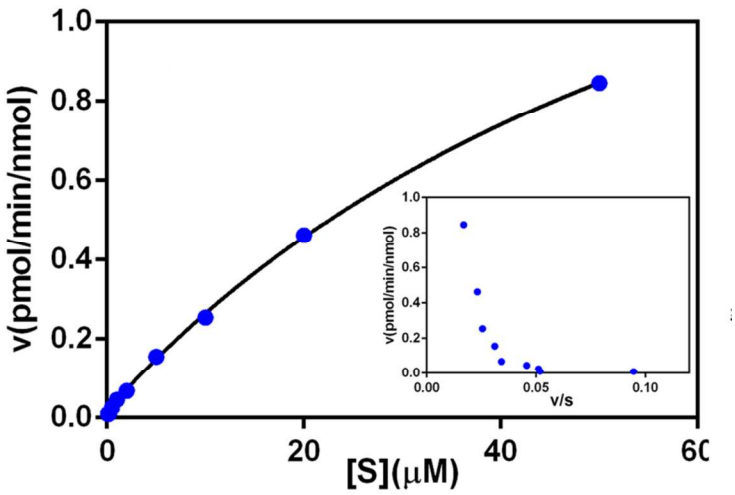


Figure 4. Plot of the initial velocity V_0 of NADE hydrolysis versus probe concentration (Inset: Eadie-Hofstee plot)

Selectivity of NADE

In addition to reactivity, another requisite to develop NADE into a functional probe for bio-imaging is selectivity. For this purpose, various potential interfering species, such as salts (K^+, Na^+), amino acids (tyrosine, glycine, glutamic acid, tryptophan, and arginine), hydrolase (alkaline phosphatase, phosphodiesterase, adenyl cyclase, creatin kinase, pepsin, trypsin, chymotrypsin), reactive oxygen species (H_2O_2 and KO_2), thiols (cystein and homocystein), physiological reductant (vitamin C), glucose, were examined. Figure 5 shows that no significant fluorescence enhancement was observed when these potential interfering species were added into NADE solution (5μM). Strikingly, this property distinguished NADE from previous SAHase probes and allows for further investigation on the utility of NADE in cells. To the best of our knowledge, such work has not yet been implemented because of the lack of efficient probes.

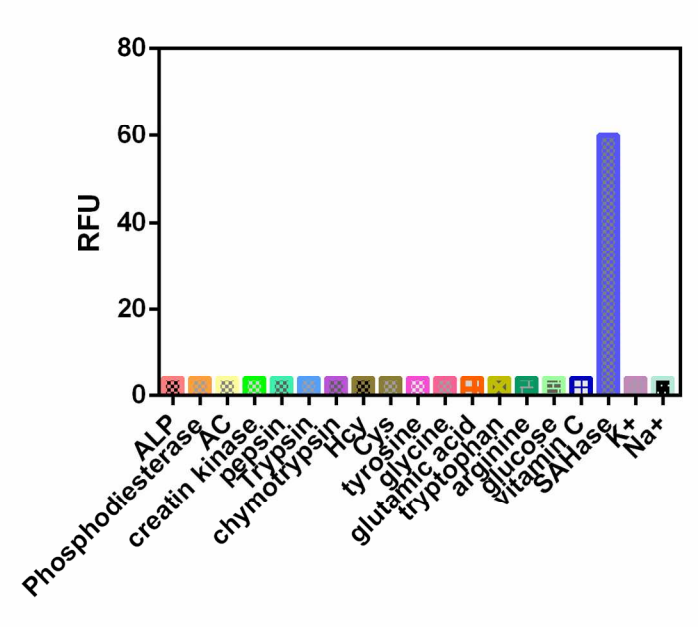


Figure 5. Fluorescence responses toward different enzymes and species (RFU=Relative Fluorescence Intensity)

Bioimaging Study in HL-60 cells

SAHase is reported to be diffused in the cytoplasm of human neutrophils[14]. To test whether NADE can measure SAHase activity in cells, we used HL-60 (human promyelocytic leukemia cells) cell lines as the research material. As an initial investigation, our initial investigation focused on two aspects: one is the specificity of NADE on SAHase when applied in a complex biological system; another is the selectivity of NADE over Hcy in vivo. Living HL-60 cells loaded with 5μM NADE for 30 min showed weak fluorescence (Figure 6b). Intracellular fluorescence was significantly enhanced when the probe-loaded cells were treated with 10 U/mL SAHase for 30 min (Figure 6a). However, a considerably weak fluorescence was observed when the probe-loaded cells were first treated with DZNep and then with SAHase (Figure 6c). These

results demonstrate that the fluorescence of HL-60 cells was specifically enhanced by SAHase. In addition, fluorescence was not enhanced by treatment with 50μM Hcy (Figure 6d), indicating that Hcy does not interfere with the determination of SAHase in living cells. Figure 6e shows the statistical analysis of NADE fluorescence in the cases above; fluorescence was considerably stronger under SAHase treatment than under inhibitor and Hcy treatments (***P<0.001). The time-dependent fluorescence intensity of NADE (Figure S10) in the four cases above is also provided in supporting information. The fluorescence of the cells costained with NADE and SYTO-59 nucleus dye revealed that the probe was metabolized by SAHase mainly in the cytoplasm (Figures S11-S14). Cell viability assay also showed that the toxicity of NADE was extremely low (IC_{50}>100μM, Figure S15). In a word, our results collectively demonstrated that NADE functioned efficiently in cells, allowing the determination of SAHase using microscopy.

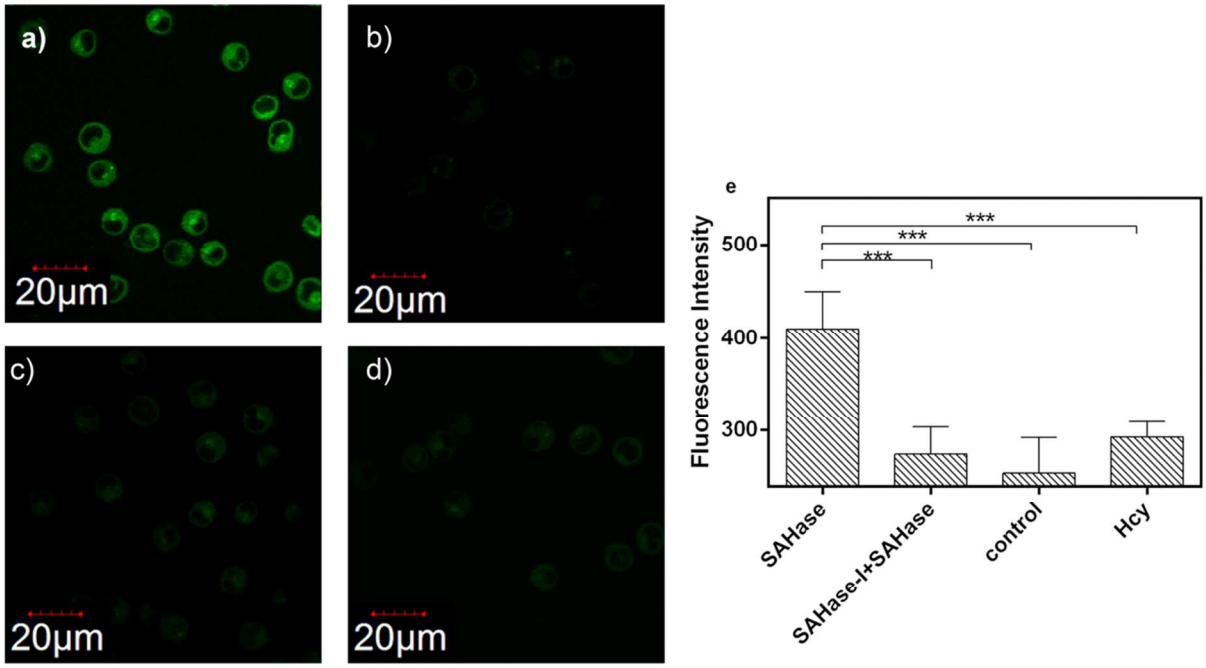


Figure 6. (a-d) Fluorescent image of NADE (5μM) incubated with HL-60+SAHase (10U/mL)/HL-60/HL-60+SAHase (10U/mL)+DZNep (10μM)/HL-60+Hcy (50μM) for 30 min (magnification: 100; excitation wavelength = 405 nm; emission wavelength = 467 nm to 517 nm). e. Statistical analyses were performed with two-sample t-test (n=7 fields of cells). ***P < 0.001, error bars are ± sem.

Inhibitor Screening Study

In addition, designing and screening SAHase inhibitors for antiviral, antiparasitic, antiarthritic, and anticancer therapeutics[36-39] have long been vigorously pursued despite the lack of a simple and convenient method for high-throughput screening (HTS). We anticipate that NADE would be well suited for the HTS of SAHase inhibitors. In this light, we tested the inhibitory effect of nine known SAHase inhibitors (structures are shown in Figure 7, Figure S8) with NADE as the substrate. We found that the probe reduced the hydrolysis rate with increasing inhibitor concentration and that the IC_{50} values of the tested inhibitors were comparable as previously reported (Figure 3c, S9, Table 1). Such results were encouraging and suggested that the HTS of SAHase inhibitors can be implemented within 20 min through this simple chemo-sensor by using only a fluorescence plate reader. Compared with previous HTS systems, this method appears to be a more advanced system in two aspects. First, the proposed method is simpler because it only needs the probe NADE and SAHase as materials, without further addition of Adohcy as the substrate or NAD as the cofactor1. Second, the use of NADE hydrolysis rate to determine SAHase activity was more accurate compared with thiol generation rate because NADE hydrolysis rate rules out the time delay of thiol reaction.

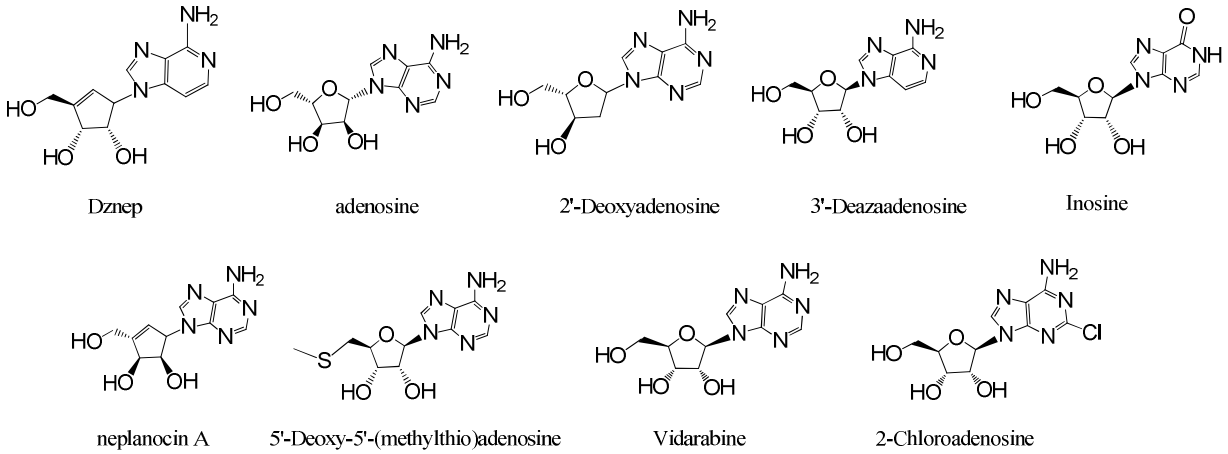


Figure 7. Structure of 9 known SAHase inhibitors

Table 1. Inhibition (IC_{50}) of SAHase by Compounds[&]

	IC_{50}(μM)	IC_{50} in literature(μM)
DZNep	0.88±1.25	0.23[40] 0.023[41](Ki)
Adenosine	8.02±1.23	
2'-Deoxyadenosine	56.77±1.05	62[42] (Ki)
3'-Deazaadenosine	11.02±1.78	4[43](Ki)
neplanocin A	0.70±1.09	0.04[44] 0.82[45]
5'-Deoxy-5'-(methylthio)adenosine	44.48±1.24	36[46](Ki)
Vidarabine	7.49±1.55	11[42] (Ki)
2-Chloroadenosine	27.37±1.15	66[42] (Ki)
Inosine	649.13±1.24	980[43](Ki)

[&]These Compounds are commercially available

Conclusion

Taken together, our data suggest that, NADE serves as a functional probe to measure SAHase activity in solutions and in living cells. NADE is an improvement of the previous SAHase fluorescent sensors, which enables researchers to directly determine SAHase activity rather than localizing the enzyme by using fluorescent antibody or quantifying the protein content by using Western blot. Moreover, given the current trend in targeted cancer therapies, NADE is expected to be useful in the ongoing search for potential anticancer drugs (SAHase inhibitors). In addition, NADE can be used as a material to reveal the enzyme mechanism of SAHase through kinetic studies. Last, this novel strategy may shed new light on the principle of drug design strategy of SAHase and can be applied in many other enzymes, especially those that catalyze sulfur-containing compounds.

■ ASSOCIATED CONTENT

Supporting Information: Synthesis, characterization, experimental details, detailed computational result, additional image data are included

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■ AUTHOR CONTRIBUTION

§ These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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