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Discovery of *S*-adenosyl-L-homocysteine hydrolase inhibitors based on non-adenosine analogs

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ABSTRACT

High throughput screening using Automated Ligand Identification System (ALIS) resulted in the discovery of a new series of *S*-adenosyl-L-homocysteine hydrolase inhibitors based on non-adenosine analogs. The optimization campaign led to very potent and competitive compound **39** with a K_i value of 1.5 nM. Compound **39** could be a promising lead compound for research to reduce elevated homocysteine levels.

Homocysteine (Hcy) is a sulfur-containing amino acid, which is an intermediate metabolite of an essential amino acid, methionine (Met). Met is condensed with ATP to form *S*-adenosyl-L-methionine (AdoMet), and AdoMet utilized as a methyl donor for methylation reactions is converted to *S*-adenosyl-L-homocysteine (AdoHcy). AdoHcy is hydrolyzed to Hcy and adenosine. There are two metabolic pathways of Hcy: (i) remethylation through methionine synthase into Met, and (ii) degradation to cysteine thorough cystathionine beta-synthase. Intracellular Hcy is highly regulated at low levels and redundant Hcy is released into the blood (see Fig. 1).

About forty years ago, McCully reported that Hcy caused vascular pathology such as arteriosclerosis and myocardial infarction.[1] Thereafter, clinical tests verified that patients having arteriosclerosis in the peripheral or cerebral vessel showed high Hcy levels.[2] In most clinical tests, a correlation between increased Hcy levels and cerebral infarction has been reported.[3–8] In a large-scale study, it has been reported that when the blood Hcy levels increases by 25% (3 μM in absolute level), the risk of coronary artery disease increases by 10%, and the risk of cerebral infarction increases by 20%;[9] and it is now suggested that Hcy is an independent risk factor. Therefore, lowering the Hcy levels could be one of the possible approaches to prevent and treat diseases such as coronary artery disease or ischemic stroke.

One of the strategies to lower the Hcy levels[10] is the inhibition of the Hcy synthetic enzyme, *S*-adenosyl-L-homocysteine hydrolase (AdoHcyase; EC 3.3.1.1.).[11] Almost all of the known AdoHcyase inhibitors are adenosine analogs.[12–14] Some of them inhibit the enzyme irreversibly, and many of them lack selectivity against related enzymes for producing adenosine, suggesting that there could remain concerns about adverse side effects.[12] A reversible inhibitor **4** has only a weak potency for the enzyme ($\sim 10^{-6}$ M) (see Fig. 2).[12] We hypothesize that reversible, competitive AdoHcyase inhibitors based on non-adenosine analogs can provide some distinct advantages in terms of selectivity and toxicity.

To explore the ligands of AdoHcyase, high-throughput screening, utilizing Automated Ligand Identification System (ALIS),[15] an affinity-based system for rapidly screening disease-associated targets, was employed.[16] ALIS screening technology based on size exclusive chromatography identifies non-covalent chemical ligands to protein targets from large combinatorial mixtures, suggesting that the ligands acquired from the system could be reversible inhibitors. Fortunately, we discovered a series of several lead candidate molecules (**5a**–**f**) illustrated in Figure 3.

The structural features of the compounds are that they contain two amides: a hydrophobic amide and a hydrophilic amide with an amine group. We addressed the replacement of the two amide moieties to improve the inhibitory activity. The general synthetic routes are described in Scheme 1.

Reaction of 1,4-dichloro-2-nitrobenzene (**6**) with 4-chlorophenol in the presence of NaH in DMF provided biphenylether **7**. Reduction of the nitro group of **7** to **8** was performed by using

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0960-894X/

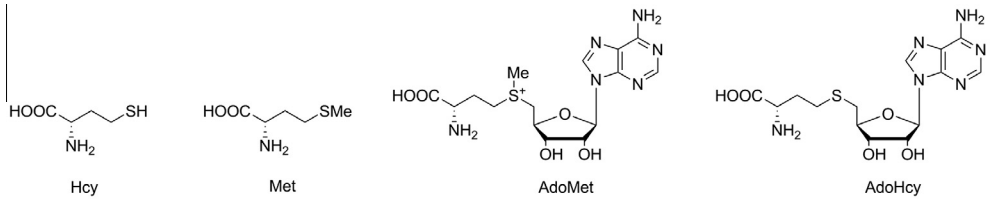


Figure 1. Hcy, Met, AdoMet, and AdoHcy.

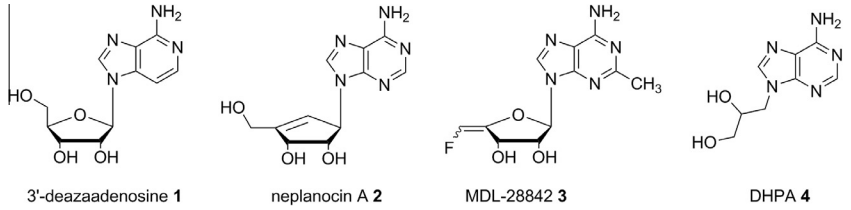


Figure 2. Known AdoHcyase inhibitors based on adenosine analogs.

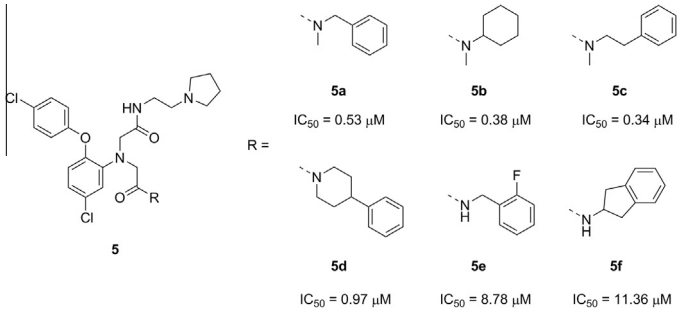
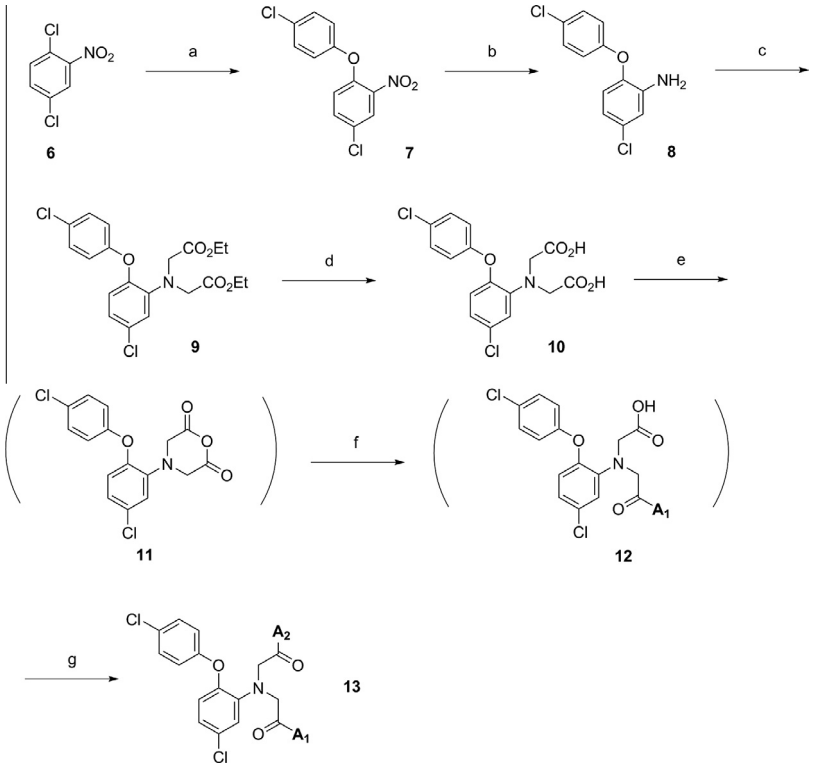


Figure 3. Representative compounds from ALIS screening and their IC_{50} values for AdoHcyase inhibition.



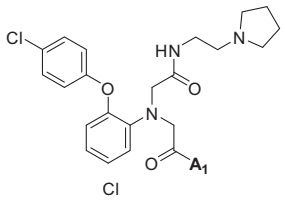
Scheme 1. Reagents and conditions: (a) 4-chlorophenol, NaH, DMF rt; (b) $FeCl_3$, charcoal activated, $NH_2NH_2{\cdot}H_2O$, MeOH, 50 °C to reflux; (c) ethyl bromoacetate, *N*,*N*-diisopropylethylamine, 140 °C; (d) 1 mol/l aq NaOH, MeOH, THF, rt; (e) EDC, DMF, rt; (f) amine A_1-H, 0 °C; (g) EDC, HOBt, amine A_2-H, 0 °C to rt.

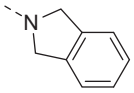
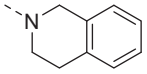
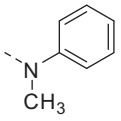
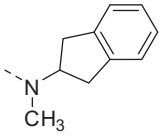
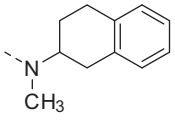
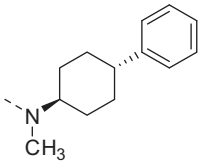
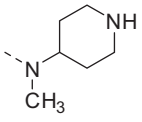
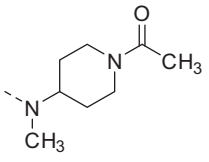
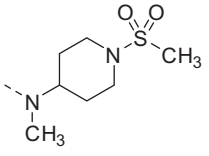
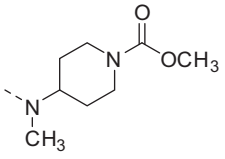
hydrazine hydrate as hydrogen sources with a catalytic amount of $FeCl_3$ and charcoal activated in MeOH under reflux conditions. Dialkylation of **8** with ethyl bromoacetate in *N*,*N*-diisopropylethylamine gave **9**, and hydrolysis of **9** with aqueous NaOH in MeOH and THF produced **10**. Compound **10** was converted to cyclic anhydride **11** by using EDC as a dehydrating agent, followed by amidation by addition of amines $\mathbf{A_1}$ provided **12**. Final amidation with amines $\mathbf{A_2}$ afforded target molecules **13**. When amines $\mathbf{A_2}$ contained protecting groups, the deprotection was performed after the amidation.

Firstly, we conducted the conversion of lipophilic amines. Because the compounds with a tertiary amide such as **5a**, **5b**, **5c**, and **5d** had more potent activities than the compounds with secondary amides such as **5e** and **5f**, we focused on the syntheses of derivatives with a tertiary amide. Results are shown in Table 1. Intramolecular cyclization of **5a** and **5c** to **14** and **15**, respectively, decreased potency, suggesting that cyclic tertiary amides are not preferable for increasing potency. Replacement of the cyclohexyl group in **5b** with a phenyl group decreased its potency (**16**). N-methylation of the secondary amide in **5f** dramatically increased the inhibitory activity against AdoHcyase (**17**). *N*-Methyl-1,2,3,4-tetrahydronaphthalen-2-amine derivative **18** had a similar strong inhibitory activity to **17**. Introduction of a phenyl group into the 4-position of the cyclohexyl group in **5b** slightly decreased its potency (**19**). Replacement of the cyclohexyl group in **5b** with piperidine led to a significant decrease in potency (**20**), but introduction of acetyl (**21**), methansulfonyl (**22**), and methoxycarbonyl (**23**) groups regained the inhibitory activity.

Secondly, we performed the modification of *N*-Me derivatives to methylhydrazine analogues (Table 2). Replacement of the indane (**17**) with an isoindoline (**24**) led to a 4-fold increase in potency, and conversion of piperidine moieties (**22**, **23**) into piperazines (**26**, **27**) also improved the inhibitory activities. On the other hand, replacement of the tetrahydronaphthalene (**18**) with a tetrahydroisoquinoline (**25**) gave a 2.5-fold loss in potency.

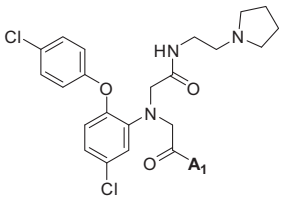
Table 1
AdoHcyase inhibition assay data of the conversion of tertiary amides

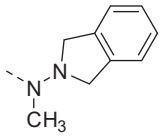
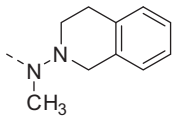
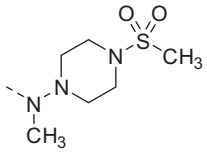
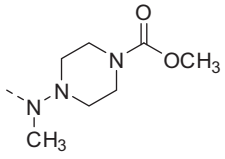


	$\mathbf{A_1}$	IC_{50} (μM)
14		>100
15		17
16		2.7
17		0.052
18		0.081
19		0.53
20		>100
21		4.6
22		0.23
23		0.89

IC_{50} values for AdoHcyase inhibition were determined in triplicates.[15]

Table 2
The modification of *N*-Me derivatives to methylhydrazine analogues



	$\mathbf{A_1}$	IC_{50} (μM)
24		0.013
25		0.20
26		0.049
27		0.044

IC_{50} values for AdoHcyase inhibition were determined in triplicates.[15]

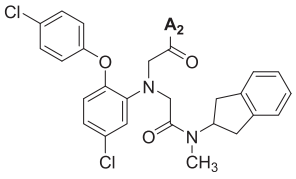
Next, we performed the conversion of hydrophilic amines with the *N*-methylindan-2-amine fixed on the lipophilic parts (Table 3). Elongation of the methylene linker to give **28** led to a 6-fold loss in potency. Replacement of the pyrrolidine with a piperidine (**29**) and a dimethylamine (**30**) showed 6 and 3 times losses in potency, respectively. The introductions of *N*-monoalkylethylenediamines such as **31**, **32**, and **34** showed comparable potency for AdoHcyase. The cyclic secondary amine series compounds **34**–**38** showed weaker inhibitory activities than **17**. Compounds **36** and **38** were more potent than **34**, **35**, and **37**, suggesting that the cyclized positions on the linker carbons may play an important role in interactions with AdoHcyase.

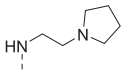
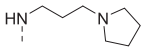
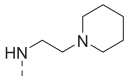
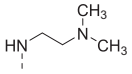
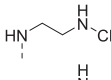
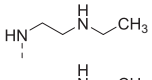
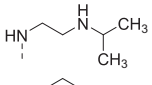
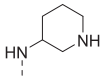
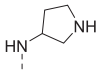
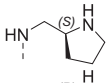
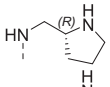
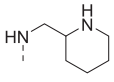
Combined with the information of SAR on the two amides parts, we synthesized compound **39**,[18] which had the strongest inhibitory activity against AdoHcyase, with an IC_{50} value of 0.0050 μM (Fig. 4).

In order to investigate the AdoHcyase inhibition pattern of this series, the kinetic analysis of **39** was performed. Lineweaver–Burk plot analysis showed that the inhibition mode of **39** was competitive with AdoHcy with a K_i value of 1.5 nM (Fig. 5).

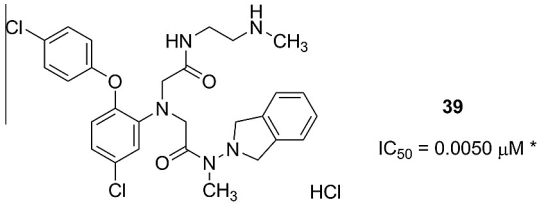
In summary, in order to explore inhibitors of *S*-adenosyl-L-homocysteine hydrolase (AdoHcyase), high throughput screening using Automated Ligand Identification System was conducted

Table 3
The conversion of hydrophilic amines



	A_2	IC_{50} (μM)
17		0.052
28		0.33
29		0.32
30		0.13
31		0.070
32		0.060
33		0.049
34		1.2
35		1.5
36		0.11
37		0.60
38		0.15

IC_{50} values for AdoHcyase inhibition were determined in triplicates.[15]



39

IC_{50} = 0.0050 μM *

* The IC_{50} value were determined in triplicates.

Figure 4. The structure and IC_{50} value of **39**.[17]

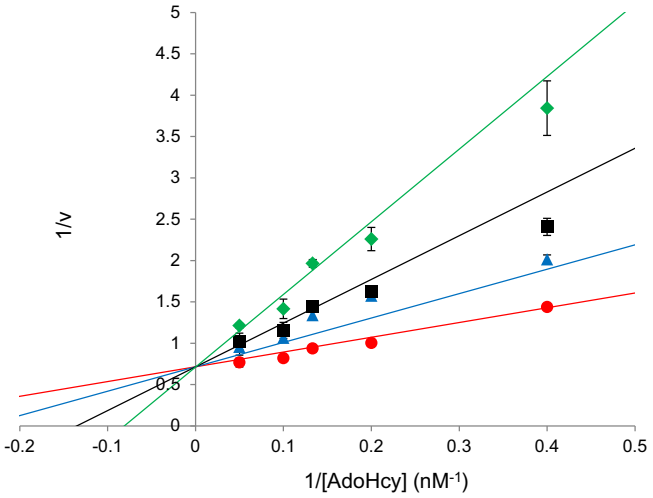


Figure 5. Lineweaver–Burk plots of AdoHcyase inhibition by **39**. Assays were conducted by incubation of various concentrations of the substrate (AdoHcy) with AdoHcyase in the absence (red circles) and presence of different concentrations [1 nM (blue triangles), 3 nM (black squares), and 6 nM (green diamonds)] of **39**. SAS software, version 9.2 (SAS Institute Inc.) was used for statistical analyses.

and we discovered a series of several lead candidate molecules. Our effort led to the discovery of the competitive inhibitor of AdoHcyase based on non-adenosine analogs **39** with a K_i value of 1.5 nM. Compound **39** could be a promising lead compound for research to reduce elevated homocysteine levels. Further lead optimization is underway and will be reported in due course.

Acknowledgments

The authors thank Dr. Kunitomo Adachi and Dr. Yoshinori Nakamura for their helpful discussions for course of this work.

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5. Coull, B. M.; Malinow, M. R.; Beamer, N.; Sexton, G.; Nordt, F.; Garmo, P. *Stroke* **1990**, *21*, 572.
6. Perry, I. J.; Morris, R. W.; Ebrahim, S. B.; Shaper, A. G.; Refsum, H.; Ueland, P. M. *Lancet* **1995**, *346*, 1395.
7. Verhoef, P.; Hennekens, C. H.; Malinow, M. R.; Kok, F. J.; Willett, W. C.; Stampfer, M. J. *Stroke* **1994**, *25*, 1924.
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10. At present, as a homocysteine-lowering therapy, ingestion of coenzymes of the metabolic enzymes such as vitamin B6, vitamin B12, and folic acid, has been tried. Ingestion of these vitamins decreases the blood Hcy levels to some extent

(Brattstrom, L. *J. Nutr.* **1996**, *126*, 1276S.). However, in a large-scale study aiming to examine an influence of vitamin therapy on the recurrence of cerebral or myocardial infarction (The Vitamin Intervention for Stroke Prevention (VISP)), a recurrence preventive effect was not observed. The cause thereof is suggested to be the absence of a sufficient decrease in Hcy by a vitamin therapy (Lowering Homocysteine in Patients With Ischemic Stroke to Prevent Recurrent Stroke, Myocarcial Infarction, and Death: The Vitamin Intervention for Stroke Prevention (VISP) Randomized Controlled Trial, Toole, J. F.; Malinow, M. R.; Chambless, L. E.; Spence, J. D.; Pettigrew, L. C.; Howard, V. J.; Sides, E. G.; Wang, C.-H.; Stampfer, M. S. *JAMA* **2004**, *291*, 565.). In this study, the vitamin therapy could lower the Hcy levels by not more than ten and several percentage.

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14. Amide derivatives as *S*-adenosyl-L-homocysteine hydrolase inhibitors based on non-adenosine analogs were reported by Tan et al. in *Chem. Pharm. Bull.* **2014**, *62*, 112. The compounds in the literature were designed and synthesized in reference to the following patent application: Nakao, A.; Suzuki, H.; Tatsumi, R.; Seki, M.; Tanaka, M.; Setsuta, T.; Iwasaki, H. WO 2009125853 (2009).
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16. ALIS screening was conducted in NeoGenesis Pharmaceuticals (later acquired by Schering-Plough) as our collaborative research program.
17. The enzyme inhibitory activity was measured using the hydrolysis activity of AdoHcy as an index. The measurement method was modification of the method of Henry H. Richards et al. (*J. Biol. Chem.* **1978**, *253*, 4476.). AdoHcy (10 μM) and adenosine deaminase (Roche) (4 units) were added to 50 mM phosphate buffer (pH 7.2, containing 1 mM EDTA) with the total amount being 200 μl, and to the solution were added a test substance and then human recombinant *S*-adenosyl-L-homocysteine hydrolase (50 ng, Diazyme Laboratories) to start the reaction, and the mixture was incubated at 37 °C for 8 min. The reaction was quenched by the addition of 1 mol/l aqueous perchloric solution (20 μl), and the mixture was centrifuged under the conditions of 10,000 rpm, 5 min, 4 °C. The supernatant was collected, and the amount of AdoHcy after the reaction was quantified by HPLC. The inhibitory rate was determined with the amount of decrease in AdoHcy before and after the reaction without using the test substance as 100%. Inhibitory rate (%) = [(amount of decrease of AdoHcy in the presence of test substance)/(amount of decrease of AdoHcy in the absence of test substance)] × 100.
18. *Spectral data of* ***39***: ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 2.49 (s, 3H), 2.78–2.87 (m, 2H), 2.83 (s, 3H), 3.25 (d, J = 6.2 Hz, 2H), 3.94 (s, 2H), 4.02 (d, J = 11.8 Hz, 2H), 4.21 (d, J = 11.8 Hz, 2H), 4.51 (s, 2H), 6.75–6.85 (m, 3H), 6.88 (d, J = 8.7 Hz, 2H), 7.25 (br s, 4H), 7.40 (d, J = 8.7 Hz, 2H), 8.50 (t, J = 5.7 Hz, 1H), 8.73 (br s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 24.21, 32.23, 34.64, 47.45, 53.85, 54.77, 56.98, 117.05, 118.68, 119.14, 122.40, 123.31, 126.31, 127.02, 128.85, 129.46, 137.34, 142.90, 143.21, 156.52, 170.49, 172.13; LC–MS: (ESI) m/z 556, 558 $[M+H]^+$; HRMS: (ESI) m/z calculated for $C_{28}H_{32}Cl_2N_5O_3$ $[M+H]^+$ 556.18767, found 556.18816.