Letter

Identification of 8-Aminoadenosine Derivatives as a New Class of Human Concentrative Nucleoside Transporter 2 Inhibitors

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Identification of 8-Aminoadenosine Derivatives as a New Class of Human Concentrative Nucleoside Transporter 2 Inhibitors

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8-aminoadenosine derivatives, human concentrative nucleoside transporter 2, dietary purines, hyperuricemia, gout

ABSTRACT: Purine-rich foods have long been suspected as a major cause of hyperuricemia. We hypothesized that inhibition of human concentrative nucleoside transporter 2 (hCNT2) would suppress increases in serum urate levels derived from dietary purines. To test this hypothesis, the development of potent hCNT2 inhibitors was required. By modifying adenosine, an hCNT2 substrate, we successfully identified 8-aminoadenosine derivatives as a new class of hCNT2 inhibitors. Compound **12** moderately inhibited hCNT2 (IC_{50} = 52 ± 3.8 μM), and subsequent structure-activity relationship studies led to the discovery of compound **48** (IC_{50} = 0.64 ± 0.19 μM). Here we describe significant findings about structural requirements of 8-aminoadenosine derivatives for exhibiting potent hCNT2 inhibitory activity.

Hyperuricemia is a condition in which serum urate levels are abnormally elevated. It is widely accepted that sustained hyperuricemia is the primary risk factor for urate deposition diseases, such as gouty arthritis, tophi, urinary stones, and renal damage.[1] Prospective cohort studies conducted in healthy men or patients with asymptomatic hyperuricemia demonstrated that serum urate levels exceeding 7.0 mg/dL are closely associated with an increased risk for developing gouty arthritis.[2,3] Moreover, a retrospective study of patients with gout revealed that the frequency of recurrent gouty arthritis decreases when serum urate levels are kept low.[4] These findings suggest the importance of proactively reducing serum urate levels to prevent the onset of gouty attacks.

Purine-rich foods have long been suspected as a major cause of hyperuricemia. Recent epidemiologic studies reported that high levels of meat and seafood consumption are associated with high levels of serum urate and an increased risk of developing gout.[5,6] Another study demonstrated that ingesting yeast RNA for several days induces hyperuricemia in healthy subjects.[7] Conversely, a low purine diet effectively lowers serum urate levels in patients with hyperuricemia.[8] Together, these findings suggest that dietary purines increase serum urate levels and suppression of the absorption of dietary purines may be a promising approach for treating hyperuricemia. Maintaining a strict diet with low purine foods over a long period is difficult, however, because nutrition is impaired and quality of life is decreased. Therefore, we attempted to develop a therapeutic drug as an alternative to restricting the intake of dietary purines.

We focused on the absorption paths of purines in the gastrointestinal tract. Dietary purines comprise various forms, such as DNA, RNA, nucleoproteins, oligonucleotides, purine nucleotides, purine nucleosides, and purine bases. Most of these forms are poorly absorbed directly via the plasma membrane because of their high molecular weights and/or highly hydrophilic nature. Thus, carrier-mediated transport likely plays an important role in the gastrointestinal absorption of dietary purines. Nucleoside transporter proteins have been extensively studied as potential carriers.[9-13] Currently, they are classified into roughly two groups in mammals.[14,15] One group contains concentrative nucleoside transporters (CNT1-3), which mediate unidirectional sodium- and/or proton-dependent transport.[16] The second group contains equilibrative nucleoside transporters (ENT1-4), which mediate bidirectional sodium-independent transport (facilitated diffusion).[17] Although distribution of the seven transporters varies depending on the tissue, in epithelia, CNTs and

ENTs are mostly localized in the apical and basolateral membranes, respectively.[14,15,18,19] Therefore, CNTs are thought to be more important carriers for the absorption of purine nucleosides in the gastrointestinal tract.

Extensive studies have revealed the inherent substrate selectivity of CNTs (Table 1).[14,15,18] CNT1 recognizes pyrimidine nucleosides and adenosine, and adenosine is transported in a high-affinity and low-capacity manner.[16,20,21] Hence, CNT1 is a pyrimidine nucleoside-specific transporter. CNT2 is the preferred purine nucleoside transporter because it transports purines and uridine.[16,22,23] CNT3 has much broader substrate selectivity, transporting both purine and pyrimidine nucleosides.[16,24] Based on these findings, CNT2 and/or CNT3 are likely to be the key transporters for the uptake of purine nucleosides in the gastrointestinal tract. Therefore, we performed quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) to confirm the expression profiles of human CNT2 (hCNT2) and CNT3 (hCNT3) in the gastrointestinal tract.[25] In the small intestine, hCNT2 was abundantly expressed, whereas hCNT3 was only weakly expressed. Furthermore, analysis of the distribution patterns in the gastrointestinal tract revealed that hCNT2 was expressed mostly in the duodenum of the upper small intestine, followed by the jejunum; and hCNT3 and hCNT2 expression were comparable in the ileum, though the expression was weak overall. These findings suggest that hCNT2 is a central carrier responsible for the absorption of purine nucleosides in the gastrointestinal tract. Hence, we selected hCNT2 as our drug target candidate and hypothesized that its inhibitors would suppress the increase in serum urate levels derived from dietary purines.

Table 1. Substrate selectivities of hCNTs.[14,15,18]

substrates		nucleoside transporters		
		CNT1	CNT2	CNT3
purine nucleosides	adenosine	T	T	T
	guanosine	NT	T	T
	inosine	NT	T	T
pyrimidine nucleosides	cytidine	T	NT	T
	thymidine	T	NT	T
	uridine	T	T	T
nucleobases		NT	NT	NT

T: transported, NT: not transported

Unlike human ENT inhibitors, there were few reports about CNT inhibitors at the beginning of our research. Among them, 2'-deoxy-5-fluorouridine,[26] phlorizin,[27,28] and 7,8,3'-trihydroxyflavone[28] are reported to be strong or moderate hCNT2 inhibitors (Figure 1). Evaluation of the inhibitory effects of these compounds on sodium-dependent inosine uptake in COS-7 cells transiently expressing hCNT2,[25] however, revealed unexpectedly weak inhibitory effects (IC_{50} > 1000 μM). Thus, to test our hypothesis, the development of more potent hCNT2 inhibitors was required. Here we describe the discovery of a new class of potent hCNT2 inhibitors along with significant findings about structural requirements for exhibiting potent hCNT2 inhibitory activity.

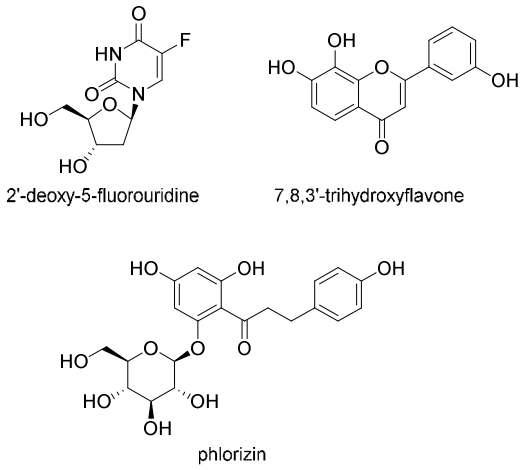


Figure 1. Structures of known hCNT2 inhibitors.

We investigated the conversion of adenosine, an hCNT2 substrate, into an inhibitor by its derivatization. In an initial study, we synthesized a series of adenosine derivatives modified at the 2-, 8-. 5'-, or N^6-position (Figure 2) and evaluated their inhibitory effects on sodium-dependent inosine uptake in COS-7 cells transiently expressing hCNT2. Among the base-modified adenosines **1**-**12**, only 8-(benzylamino)adenosine **12** exhibited an inhibitory effect greater than 50% at 100 μM (IC50 = 52 ± 3.8 μM). On the other hand, the sugar-modified adenosines **13**-**24** were inactive or almost inactive (IC50 > 100 μM), consistent with a previous report demonstrating that the 5'-hydroxy group in the ribofuranosyl moiety is essential for recognition by hCNT2.[29] Thus, we performed structure-activity relationship (SAR) studies of the 8-modified adenosines.

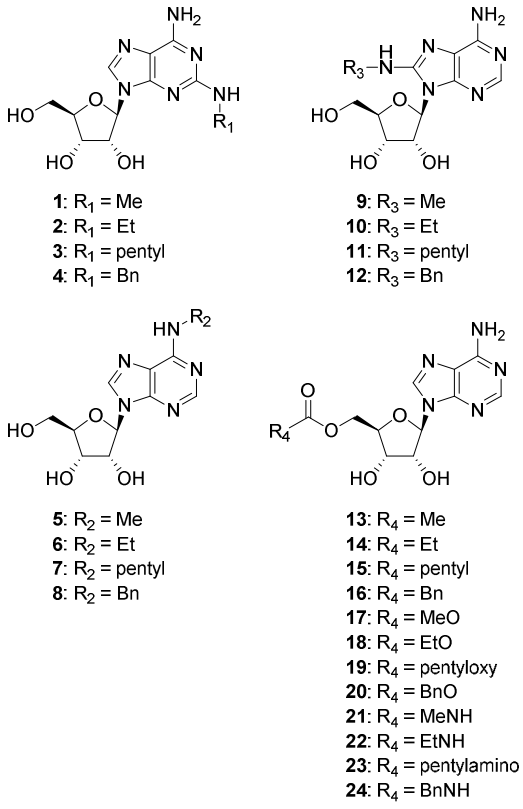
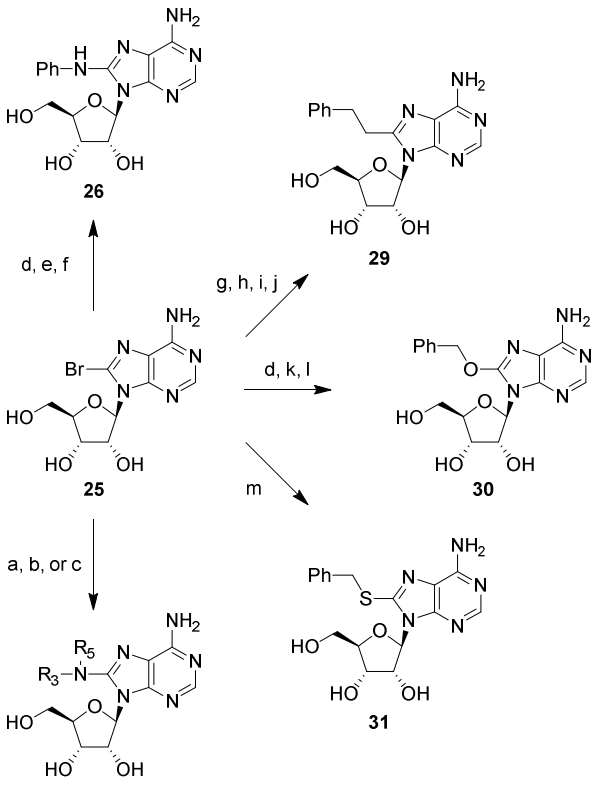


Figure 2. Structures of modified adenosines evaluated in an initial study.

The 8-substituted adenosines evaluated in this study were synthesized from commercially available 8-bromoadenosine **25** as a common starting material (Scheme 1). Aromatic nucleophilic substitution reactions of compound **25** at the 8-position with appropriate amines and BnSH gave 8-aminoadenosine derivatives **9**-**12**, **27**, **28**, **32**-**48** and 8-(benzylsulfanyl)adenosine **31**, respectively.[30] *O*-Silylation of compound **25** and subsequent Pd-catalyzed coupling reaction with PhNH2 followed by treatment with NH_4F gave **26**. *O*-Acetylation of compound **25** and subsequent Sonogashira coupling with ethynylbenzene gave 2',3',5'-tri-*O*-acetyl-8-(phenylethynyl)adenosine, which was then reduced under catalytic hydrogenation condition before treatment with NaOMe to afford **29**. *O*-Silylation of compound **25** and subsequent aromatic nucleophilic substitution reaction at 8-position with BnOH followed by treatment with NH_4F gave **30**.

Scheme 1. Synthesis of 8-substituted adenosines[a]



27: R_3 = 2-phenylethyl, R_5 = H
28: R_3 = 3-phenylpropyl, R_5 = H
32: R_3 = Bn, R_5 = Me
33: R_3 = (*R*)-1-phenylethyl, R_5 = H
34: R_3 = (*S*)-1-phenylethyl, R_5 = H
35: R_3 = 2-methylbenzyl, R_5 = H
36: R_3 = 3-methylbenzyl, R_5 = H
37: R_3 = 4-methylbenzyl, R_5 = H
38: R_3 = 2-chlorobenzyl, R_5 = H
39: R_3 = 3-chlorobenzyl, R_5 = H
40: R_3 = 4-chlorobenzyl, R_5 = H
41: R_3 = 2-methoxybenzyl, R_5 = H
42: R_3 = 3-methoxybenzyl, R_5 = H
43: R_3 = 4-methoxybenzyl, R_5 = H
44: R_3 = 1-naphthylmethyl, R_5 = H
45: R_3 = 2-naphthylmethyl, R_5 = H
46: R_3 = biphenyl-2-ylmethyl, R_5 = H
47: R_3 = biphenyl-3-ylmethyl, R_5 = H
48: R_3 = biphenyl-4-ylmethyl, R_5 = H

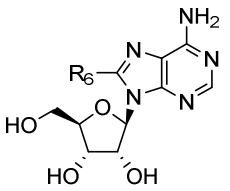
[a]Reagents and conditions: (a) aqueous R_3R_5NH, EtOH, 100 °C, in a sealed tube; (b) R_3R_5NH, *i*-Pr_2NEt, EtOH or PrOH, 120 °C, in a sealed tube; (c) R_3R_5NH, *i*-Pr_2NEt, EtOH, 150 °C, in a sealed tube, microwave irradiation; (d) *tert*-butyldimethylsilyl chloride, imidazole, DMF, ambient temp.; (e) PhNH2, tris(dibenzylideneacetone)dipalladium(0), *tert*-BuONa, (±)-BINAP, toluene, 80 °C; (f) NH_4F, MeOH, reflux; (g) Ac_2O, Et_3N, 4-dimethylaminopyridine, MeCN, ambient temp.; (h) ethynylbenzene, $(Ph_3P)_4Pd$, CuI, Et_3N, DMF, 80 °C; (i) H_2, 10% Pd-C, AcOEt, ambient temp.; (j) NaOMe, MeOH, ambient temp.; (k) BnOH, *tert*-BuOK, 40 °C then (l) NH_4F, MeOH, reflux; (m) BnSH, *i*-Pr_2NEt, EtOH, 120 °C, in a sealed tube.

The inhibitory effects of analogs of **12** were investigated (Table 2). Both deletion and elongation of the methylene linker between the nitrogen and benzene ring decreased the inhibitory activity (compound **12** vs **26**-**28**). Replacement of the nitrogen with carbon, oxygen, or sulfur was also disadvantageous (compound **12** vs **29**-**31**). Methylation of the nitrogen or at the benzylic position led to a complete loss of activity (compound **12** vs **32**-**34**). These findings indicate that, in the 8-substituent of compound **12**, the methyleneamino (-CH2NH-) structure was essential for the inhibitory effect, and therefore we performed SAR studies on derivatives of compound **12** by modifying the terminal phenyl moiety (Table 3). Introduction of a substituent at the 3- or 4-position on the benzene ring improved the inhibitory activity regardless of the electronic nature (compound **12** vs **36**, **37**, **39**, **40**, **42**, and **43**). In contrast to the 2-chloro derivative **38**, both the 2-methyl derivative **35** and 2-methoxy derivative **41** were less active than compound **12**, suggesting a spatial limitation around the position in the binding mode. An electron-withdrawing group on the benzene ring might be preferred because all chloro derivatives exhibited more potent activity than the corresponding methyl or methoxy derivatives. On the other hand, replacement of the phenyl group with a 1- or 2-naphthyl group also enhanced the inhibitory activity (compound **44** and **45**), regardless of the substituted position. Based on these results, a space around the phenyl moiety that accommodates rather large substituents might be present in the binging mode. Therefore, we designed biphenyl derivatives **46**-**48**. The biphenyl-2-yl derivative **46** was inactive, as expected. The biphenyl-3-yl derivative **47** exhibited 3-fold more potent inhibitory activity than compound **12**, but the activity did not surpass naphthyl derivatives **44** and **45**. The introduction of a biphenyl-4-yl group, however, was highly effective, and the biphenyl-4-yl derivative **48** exhibited 81-fold more potent inhibitory activity than the parent compound **12**. In addition, compound **48** exhibited inhibitory activity 1500-fold more potent than that of 2'-deoxy-5-fluorouridine, phlorizin, and 7,8,3'-trihydroxyflavone, which are well-known hCNT2 inhibitors.

Finally, we performed a conformational analysis of the most potent compound **48** by the density functional theory (DFT) B3LYP/6-31g* method in Spartan'10 to get insight into the bioactive conformation. The calculations demonstrated that compound **48** is likely to take predominantly the *anti* conformation rather than the *syn* conformation, of which energy difference was 3.27 kcal/mol.[31] The preference for the *anti* conformers to the *syn* conformers would be due to the intramolecular hydrogen bonding formed by the N-H of the 8-substituent with the 5'-hydroxy oxygen and the ribose ring oxygen. These conformational analysis results are in accord with structure-activity relationship results that the N-H moiety in the 8-substituents is essential for exhibiting the inhibitory activity against hCNT2.

In conclusion, we identified a new class of potent hCNT2 inhibitors. Starting with a natural substrate, adenosine, we successfully identified 8-aminoadenosine derivative **12** and subsequent SAR studies led to the discovery of compound **48** (IC50 = 0.64 ± 0.19 μM). Conformational analysis data of compound **48** suggested the preference for anti conformers, and therefore valuable information for speculating the bioactive conformation was provided. The inhibitor **48** can be a prototype compound for the development of conceptually new drugs for the treatment of hyperuricemia and gout.

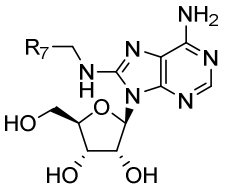
Table 2. Inhibitory Effects on Sodium-dependent Inosine Uptake in COS-7 Cells Transiently Expressing hCNT2.



compd	R_6	Inh.%[a]	IC_{50} (μM)[b]
12	$PhCH_2NH$-	58	52 ± 3.8
26	PhNH-	36	289 ± 87
27	$Ph(CH_2)_2NH$-	39	233 ± 19
28	$Ph(CH_2)_3NH$-	26	536 ± 89
29	$Ph(CH_2)_2$-	<5	-
30	$PhCH_2O$-	<5	-
31	$PhCH_2S$-	<5	-
32	$PhCH_2N(Me)$-	<5	-
33	(*R*)-PhCH(Me)NH-	<5	-
34	(*S*)-PhCH(Me)NH-	<5	-
2'-deoxy-5- fluorouridine		17	-
phlorizin		14	-
7,8,3'-trihydroxyflavone		<5	-

[a]Inhibition% at 100 μM. If more than 20% inhibition was observed, the IC50 value was calculated. [b]Concentration of each compound required to inhibit inosine uptake by 50%. Data represent mean ± standard error of the mean of at least three independent experiments unless otherwise stated.

Table 3. Inhibitory Effects on Sodium-dependent Inosine Uptake in COS-7 Cells Transiently Expressing hCNT2.



compd	R_7	IC_{50} (μM)[a]
12	phenyl	52 ± 3.8
35	2-methylphenyl	65 ± 5.9
36	3-methylphenyl	19 ± 2.3
37	4-methylphenyl	11 ± 4.2
38	2-chlorophenyl	5.7 ± 1.2
39	3-chlorophenyl	5.4 ± 1.3
40	4-chlorophenyl	11 ± 2.8
41	2-methoxyphenyl	>100
42	3-methoxyphenyl	13 ± 5.7
43	4-methoxyphenyl	36 ± 13
44	1-naphthyl	5.7 ± 1.1
45	2-naphthyl	5.3 ± 1.7
46	biphenyl-2-yl	>100
47	biphenyl-3-yl	21 ± 9.8
48	biphenyl-4-yl	0.64 ± 0.19
2'-deoxy-5- fluorouridine		>1000[b]
phlorizin		>1000[b]
7,8,3'-trihydroxyflavone		>1000[b]

[a]Concentration of each compound required to inhibit inosine uptake by 50%. Data represent mean ± standard error of the mean of at least three independent experiments unless otherwise stated. [b]Inhibition was less than 50% at 1000 μM.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and characterization data of all synthesized compounds, assay method, and conformational analysis data of compound **48**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

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ABBREVIATIONS

RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; RT-PCR, reverse transcription-polymerase chain reaction

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(30) In case of compound **34**, an additional protection-deprotection step was needed to obtain the compound in pure form.
(31) Figure of the most stable *anti* and *syn* conformers were provided in Supporting Information.

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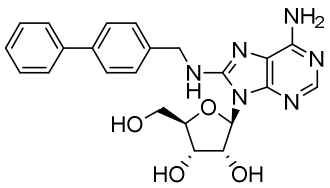

Compound 48
hCNT2 IC_{50} = 0.64 ± 0.19 μM